PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, G01N 33/566, C12N 15/12, 5/10

A1

(11) International Publication Number: WO 96/41013

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09638

(22) International Filing Date: 6 June 1996 (06.06.96)

(30) Priority Data:
08/482,436 7 June 1995 (07.06.95) US

(71) Applicant: LIGAND PHARMACEUTICALS INCORPORATED [US/US]; 9393 Towne Centre Drive, San Diego, CA 92121 (US).

(72) Inventors: McDONNELL, Donald, P.; 10382 Rue Riviera Verte, San Diego, CA 92131 (US). TZUKERMAN, Maty; 25220 Kibbutz Lohamei Hatettaot (IL). DELORME, Evelyn; 4458 Benhurst Avenue, San Diego, CA 92122 (US). MINER, Jeffrey, N.; 4572 Pauling Avenue, San Diego, CA 92122 (US). GLEESON, Martin, A., G.; 12458 Carmel Cape, San Diego, CA 92130 (US). WEN, Xiaohong, Dawn; 5137-A Renaissance Drive, San Diego, CA 92122 (US). PIKE, J., Wesley; 911 Springwood Lane, Encinitas, CA 92024 (US).

(74) Agents: CHEN, Anthony, C. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR SCREENING FOR RECEPTOR AGONISTS AND ANTAGONISTS

(57) Abstract

This invention relates to a method for identifying a receptor agonist or antagonist by providing a nucleic acid encoding a receptor having a first TAF region able to activate transcription from a promotor, and a second TAF region mutated to have the functional context of the second TAF region, but not able on its own to activate transcription of the promoter. The nucleic acid is provided within a cell unable to exhibit transcription from the promoter in the presence of the second TAF region alone, but able to exhibit transcription from the promoter in the presence of the first TAF region. The cell further includes a reporter construct containing the promoter operatively linked to a reporter gene. The reporter gene is transcribed when the promoter is activated in the presence of the first TAF region. The method further includes contacting the cell with a potential agonist or antagonist, under conditions in which contact of the cell with a known agonist or antagonist of the receptor increases or decreases transcription from the promoter and the level of the product of the reporter construct. Finally, the method involves measuring the level of increase of the product of the reporter construct as an indication of the agonist activity of the potential agonist. The method is applicable to intracellular receptors including ER, AR, PR and GR.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

DESCRIPTION

Method For Screening For Receptor Agonists And Antagonists

Related Applications

This application is a continuation-in-part of McDonnell and Tzukerman, entitled "Method For Screening For Receptor Agonists", filed April 6, 1994, U.S. Serial No. 08/223,943, which is a continuation-in-part of McDonnell and Tzukerman, entitled "Method For Screening For Receptor Agonists", filed January 10, 1994, U.S. Serial No. 08/179,750, which is a continuation-in-part of McDonnell and Tzukerman, entitled "Method For Screening For Receptor Agonists", filed April 4, 1993, U.S. Serial No. 08/045,807, incorporated by reference herein, including the drawings attached thereto.

Field of the Invention

This invention relates to methods and constructs useful for identifying agonists and antagonists active at intracellular receptors.

Background of the Invention

Steroid hormones, such as estrogen, progesterone, androgens, glucocorticoids, and mineralocorticoids travel via the blood stream to their target cells, enter these cells, and then bind to steroid hormone receptors. The steroid hormone receptors exist in inactive apoprotein forms either in the cytoplasm or nucleus. Upon binding their respective hormonal ligands, the receptors become activated. The activated receptor can bind effectively to a hormone response element (HRE) on a chromosome and activate transcription of a *cis*-linked gene.

The steroid hormone receptor superfamily includes receptors for the steroids, e.g., estrogen,

progesterone, glucocorticoid, mineralocorticoid and androgen. It also includes receptors for thyroid hormone, vitamin D, retinoic acid and a 9-*cis* retinoid acid and ecdysone. Furthermore, it includes a large number of proteins having sequence homologous to the steroid hormone receptors, but whose ligands are unknown, e.g., peroxisome proliferator activated receptor. These proteins have been termed "orphan receptors".

A typical steroid hormone receptor can be divided into six domains, A, B, C, D, E and F as indicated in Figure 11. The function of each domain is indicated by solid lines. The N-terminal A/B domain contains a transactivation function. The C region is responsible for DNA binding and receptor dimerization. The D region is a hinge region which allows the protein to bend or alter conformation. The E region is important for dimerization, transactivation, intramolecular repression and ligand binding. DNA sequences responsive to steroid hormones have been termed hormone response elements (HRES).

Evans et al., U.S. Patent 5,071,773, incorporated by reference herein, describes an assay by which hormone receptors, ligands for such receptors, and proteins having transcription activating properties of a hormone receptor, can be detected. Generally, the assay involves using a cell containing both a DNA encoding a receptor protein, and a DNA encoding a hormone responsive element (e.g., a promoter) linked to an operative reporter gene. When a suitable hormone or ligand is provided to the cell, a receptor-hormone is formed and delivered to an appropriate DNA-binding region to thereby activate the hormone responsive element and cause expression of the reporter gene. The expression product of the reporter gene is detected by standard procedures known to one skilled in the art.

Webster et al., Cell 54:199 (1988), used chimeric receptors to localize regions responsible for transcription activation function. The authors propose that a hormone is responsible for allowing a receptor to recognize a DNA response element, and that the hormone induces a transcription activation function in the hormone-binding domain.

Summary of the Invention

The present invention features a method for identifying agonists and antagonists of an intracellular receptor. These agonists and antagonists modulate the transcription activity of a promoter through a TAF region of the receptor in a cell. The present invention also features a method for using these agents to treat diseases and pathological conditions affected by an intracellular receptor, such as, but not limited to, breast cancer, endometrial cancer, fibroids, and endometriosis. This invention makes it possible to screen large collections of natural, semisynthetic, or synthetic compounds for therapeutic agents that affect the transcription activation activity of an intracellular receptor.

This invention provides an assay to screen for an agonist or antagonist of an intracellular receptor which interacts with one of the TAF regions of the receptor. Not only can an agonist or antagonist be specifically identified, but the type of agonist or antagonist can be determined in such an assay. The agonists and antagonists so identified may be used to selectively modulate a promoter in a cell.

In order to detect agonists and antagonists which act through a particular TAF region of a chosen intracellular receptor, Applicant inactivates the transcription activation function of other TAF regions in the receptor. Considering that the activity of a TAF region is dependent on having the functional context of

other TAF regions available, this invention describes introducing mutations to receptor constructs to inactivate the transcription activation activity of these other TAF regions, while retaining the functional context of these TAF regions. Such modified receptors can be used to identify promoter and cell-type specific requirements for the transcriptional activation activity of a particular TAF region. This invention allows the determination of such promoter-type and cell-type specific differences in transcription activation activity.

This invention also relates to selecting a cellular context in which the remaining TAF region is able to activated transcription from a promoter. Furthermore, this invention relates to selecting a promoter context which is responsive to the transactivation by the remaining TAF region. Therefore, by properly preparing a receptor construct and selecting a cellular context and promoter context, the claimed assay allows the remaining TAF region to exhibit its transcription activation activity on a promoter in a cell.

Without being bound by any theory, Applicant proposes that such promoter-type and cell-type specific transcription activity of a TAF region may be explained by a model in which one TAF region acts as a dominant transcriptional activator, and a second TAF region as a transcriptional facilitator (see Fig. 7). The second TAF region prepares the transcription apparatus for the action of the first TAF region. Such preparation may be recruitment of basic transcription factors, alteration of chromatin structure, or causing removal of a transcriptional repressor. Alternatively, the second TAF region may prepare a transcription apparatus for other transcriptional activators, and alone have little inherent transcription activation activity. In such a model, the first TAF region is unable to access the transcription apparatus until the second TAF region has

acted appropriately to prepare it for the action of the first TAF region.

Applicant proposes that cell specificity for a particular TAF activity may reflect the presence or absence of a function in a cell that mimics the presence of a helper TAF region, respectively. Such a mimetic in a cell will allow a receptor construct having a mutated and inactive helper TAF region to be active because the inactive portion of the receptor is complemented by the active functionality present in the cell.

A similar model may exist for promoter specificity, i.e., only selected promoters will be activated by a particular TAF in a cell, depending on the functionalities present in those promoters. In this model, the difference in agonist activity of various agents is dependent on the effect of that agonist on the TAF1 region or TAF2 region, and interaction of the resulting TAF1 or TAF2 region with a selected promoter or general transcription apparatus.

Thus, in a first aspect, the invention features a method for identifying an agonist or antagonist of an intracellular receptor. The method includes providing a cell containing a nucleic acid encoding an intracellular receptor having a first TAF region and a second TAF region. The first TAF regions is able to activate transcription from a selected promoter, and the second TAF region is mutated so that, while it provides the functional context of that region, it is not able to activate transcription from the promoter.

The cell also includes a reporter construct which has a promoter region which is activated to cause transcription of a reporter gene in the presence of a receptor having an active TAF region corresponding to that which is not mutated above. The promoter is not activated by the presence of a receptor containing only the TAF region corresponding to that mutated above. The

receptor construct and reporter construct may be inserted in two vectors or a single vector.

The cell is so chosen that no, or minimal, transcription of the promoter occurs in the presence of a receptor having only an unmutated second TAF region corresponding to that mutated above (and not the other TAF region). The cell is also chosen such that transcription of the promoter occurs in the presence of a receptor having the above nonmutated first TAF region alone.

For example, in a receptor construct having an operative TAF1 region and a mutated, inoperative TAF2 region, transcription of the promoter will not occur (i.e., no significant level of transcription is detectable, usually less than 5-10% of normal levels) in the presence of a receptor having an operative TAF2 region only, but will occur in the presence of a receptor having an operative TAF1 region only.

The method further includes the step of contacting the cell with a potential agent under conditions in which contact of the cell with a normal agonist (e.g., estrogen for an estrogen receptor) or antagonist will either increase or decrease the transcription of the reporter gene from the promoter, respectively. The method may involve transcribing the reporter construct at a basal (low or minimal) level in the cell before the agonist or antagonist is applied. Alternatively, the method may involve applying the agonist or antagonist first, and then transcribing the reporter construct.

Finally, the method involves the step of measuring the level of increase or decrease of the reporter gene product, as an indication of the agonist or antagonist activity of said agent, respectively.

By "intracellular receptor" is meant an transcription polypeptide in the cytoplasm or nucleus of a cell whose transcription regulation activity is regulated by binding of small molecules such as steroid

hormones, including, but not limited to, estrogen receptor (ER), retinoid acid receptors (RAR), retinoid X receptors (RXR), glucocorticoid receptors (GR), progesterone receptors (PR), androgen receptors (AR), mineralocorticoid receptor (MR), thyroid hormone receptors (TR), peroxisome proliferator activated receptor (PPAR), and vitamin D receptors. An intracellular receptor may be mutated by site-directed mutagenesis, deletion, substitution, and other genetic methods known to those skilled in the art. The intracellular receptor may either be endogenous to the cell or transfected into the cell.

By "transcription polypeptide" is meant a cytoplasmic or nuclear protein that, when activated, binds a promoter, enhancer or silencer either directly, or indirectly through a complex of proteins to modulate the transcription activity of the promoter.

By "TAF" is meant a transactivation function domain located in an intracellular receptor having the ability to interact with a transcription target and activate the transcription from a promoter. In the art, a TAF region sometimes is referred to as an AF region. The A/B domain and E domain of a typical steroid hormone receptor contain TAF regions. Other TAF regions may be identified by deletions, site-directed mutagenesis and other methods known to those skilled in the art.

By "agonist" is meant a compound or composition which when combined with an intracellular receptor stimulates or increases a reaction typical for the receptor, e.g., transcription activation activity.

By "antagonist" is meant a compound or composition which when combined with an intracellular receptor interferes or decreases a reaction typical for the receptor, e.g., transcription activation activity.

By "promoter" is meant a DNA regulatory region proximal to the RNA start site in the 5' or upstream direction capable of binding directly or indirectly to

RNA polymerase and associated transcription factors in a cell and initiating transcription of a downstream (3' direction) coding sequence. A promoter of a DNA construct, including an oligonucleotide sequence according to the present invention, may be linked to a heterologous gene where the presence of the promoter influences transcription from the heterologous gene, including genes encoding reporter molecules such as human growth hormone, luciferase, chloramphenicol acetyl transferase, β-galactosidase, secreted placental alkaline phosphatase and other secreted enzyme reporters.

By "reporter gene" is meant a nucleotide sequence encoding a polypeptide whose presence or activity is readily detectable, including, but not limited to, luciferase, chloramphenicol acetyl transferase (CAT), β-galactosidase, secreted placental alkaline phosphatase, human growth hormone, and other secreted enzyme reporters. Generally reporter genes encode a polypeptide not otherwise produced by the host cell which is detectable by *in situ* analysis of the cell culture, e.g., by the direct fluorometric, radioisotopic or spectrophotometric analysis of the cell culture without the need to remove the cells for signal analysis from the culture chamber in which they are contained. Preferably the gene encodes an enzyme which produces colorimetric or fluorometric changes in the host cell which is detectable by *in situ* analysis and which is a quantitative or semi-quantitative function of transcriptional activation. Exemplary enzymes include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes whose function can be detected by appropriate chromogenic or fluorogenic substrates known to those skilled in the art.

By "functional context" is meant only a few amino acids (i.e., up to 10), or preferably only 1-3 amino

acids are altered in one region so that the interaction of a hormone or transcription factor with the region is altered to a minimum extent (preferably, the interaction is unaltered). Such interaction will allow full expression of the activity of the unaltered or non-mutated region. Thus the functional context of one TAF region contains the functional activities of the other TAF region with respect to agonist binding, dimerization, and heat shock protein interaction, but not with respect to the ability to activate transcription.

In another word, functional context is meant the part of a TAF region that contains the functional activities of another TAF region with respect to agonist binding, dimerization, and heat shock protein interaction, but not with respect to the ability to activate transcription. The functional context of a TAF region can be preserved while the transcription activation activity of the TAF region is destroyed. For example, receptor dimerization or the interaction of a hormone or transcription factor with the TAF region is altered to a minimum extent, or preferably unaltered, when certain mutations are made in the TAF region (e.g., a few amino acids (i.e., up to 10), or preferably only 1-3 amino acids are mutated in the TAF region). Such interaction allows the fullfillment of transcription activation activity of another unaltered or non-mutated TAF region.

In this invention, a cell is provided with a specific receptor construct having a selected TAF activity, and having a suitable promoter, which responds to the TAF activity, linked to an operative reporter gene. The promoter is selected in conjunction with a specific cell so that activity of an agonist or antagonist is observed only under selected conditions. Thus, in one example, the receptor may have an active first TAF region, and a mutated (inactive) second TAF

region which provides the functional context for the first TAF region, and the cell is chosen such that it has a component which mimics or replaces the second TAF region function of the receptor at the chosen promoter. The promoter in turn provides an appropriate binding context to allow the component to manifest the desired TAF functions. In this way, an agonist or antagonist which acts at the first TAF region can be readily identified by its ability to increase or decrease expression of the reporter gene, despite the lack of an active second TAF region on the receptor.

In preferred embodiments, the agent is a human hormone agonist or antagonist, and a nuclear receptor, e.g., a human hormone receptor is encoded by the nucleic acid within the cell.

In another preferred embodiment, the receptor has a mutated TAF2 region, and the cell and promoter are chosen to exhibit no, or minimal, response to the presence of TAF2 alone.

In a further preferred embodiment, such a cell is a liver cell (specifically, a HepG2 cell) in which a receptor with an operative TAF2 region has no activity. That is, there is no inherent transcriptional activity with a receptor having just TAF2 and no TAF1 region present in the cell, but there is transcriptional activity with a receptor only having an operative TAF1 region available. Most preferably, the promoter (e.g., a C3 promoter) is chosen such that it does not require a receptor with a TAF2 function to be provided within the chosen cell, so that any agonist (e.g., an ER agonist) which acts in conjunction with a functional TAF1 in the receptor construct is able to show its agonist activity.

In yet another preferred embodiment, the candidate agent is selected from the group consisting of glucocorticoids and other agonists and antagonists of GR, nonsteroid glucocorticoids; estrogens and other agonists and antagonists of ER, nonsteroid estrogens;

androgens and other agonists and antagonists of AR, nonsteroid androgens; progestins and other agonists and antagonists of PR; non-steroid progestins; mineralocorticoids and other agonists and antagonists of MR, nonsteroid mineralocorticoids.

Candidate compounds include but are not limited to those disclosed and referred to in Table 2. Peptide or small molecule combinatorial libraries can be used to screen for agonists and antagonists (Bunin, B.A.N. Ellman, J. A., *J. An. Chem. Soc.* 114:10997-10998 (1992) and references contained therein).

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

In another aspect, the invention features a method for identifying a receptor agonist by providing a cell containing both a nonmutated intracellular receptor having functional TAF1 and TAF2 regions and a reporter construct having a promoter.

The cell is chosen to lack a mimicking TAF1 or TAF2 activity (i.e., a receptor having either an active TAF1

or TAF2 region alone does not cause activation of transcription in the cell). The promoter is chosen so that activation is achieved in this cell from this promoter in the presence of an agonist for the receptor which acts only through one of the TAF regions but not both.

In a preferred embodiment, a liver cell (e.g., HepG2) and a complex C3 promoter together provide a useful assay for agonists (e.g., an ER agonist) active at a TAF1 region only. The liver cell and C3 promoter have a TAF2 activity, but the promoter is not activated in the presence of a receptor having an active TAF2 region alone. However, the promoter is active in the presence of an active TAF1 region. Thus, agonists active at the receptor TAF1 region can be identified as those which increase expression of the reporter gene.

In another preferred embodiment, the TAF2 region is mutated.

This cell provides a useful screening test to determine the type of agonist tested. The level of transcription observed is related to the agonist type as exemplified below. The above two methods (with mutated and nonmutated receptor constructs) may be used in combination to detect, grade or type agonists at a selected receptor.

Unlike prior methods in which laborious procedures were involved to detect useful agonists, the methods described herein allow rapid screening of potential agonists and antagonists of intracellular receptors, including, but not limited to, estrogen, progesterone, glucocorticoid, androgen and mineralocorticoid receptors. The assay may be conducted not only in the human derived cells, but also in other eucaryotic cell lines, such as chicken and yeast cell lines.

The agonists and antagonists identified by this invention have advantages in treating diseases. For example, an estrogen agonist can be identified which is

useful for treatment of osteoporosis. In osteoporosis, it appears as though TAF1 activity alone is sufficient for prevention of bone loss. Thus, agonists having activity only at the TAF1 region and not at the TAF2 region of the receptor are useful for disease treatment with no or fewer side effects.

In another aspect, the invention features a method for treating or preventing an estrogen related disease or condition. By "estrogen related disease or condition" is meant a disease or condition that is caused or associated with an elevated or depressed level of the hormone estrogen, including, but not limited to, osteoporosis, breast cancer, uterine cancer, endometriosis, vasomotor abnormalities, hot flashes, depression, other psychiatric abnormalities and uterine fibroids. In some diseases the patient may be unable to produce estrogen in an amount required by the body. In other diseases, estrogen may be overproduced. By "hormone" is meant a naturally occurring biochemical that will function as a receptor agonist. Synthetic hormones are more properly referred to as agonists.

The method involves administering a chemical compound other than keoxifene, but having a keoxifene-like transcriptional profile to a patient (or causing <u>in vivo</u> production of a compound other than keoxifene, but having a keoxifene-like transcriptional profile).

The treatment may have the effect of preventing new tumors from developing and/or of shrinking the size of existing tumors. The method includes varieties of hormone replacement therapy. In this method, the patient is preferably first identified as suffering from such a disease or condition by standard techniques, and then treated as described below.

By "keoxifene-like transcriptional profile" is meant the activity of a compound in producing a normalized response similar to that produced by keoxifene, i.e., a relatively low TAF1 response at low concentrations of

the compound but relatively high response at higher concentrations of the compound. In addition, little or no TAF2 response should be present at all concentrations. Furthermore, it should have a greater (about twice or more) TAF1 response than with a wild type receptor at higher concentrations (about 10^{-7} M), see Fig. 8E compared to Figs. 8A-D). The administration of compounds with keoxifene-like transcriptional profiles is expected to exhibit bone protecting activity and uterine/breast sparing activity.

By "bone protecting activity" is meant the ability to prevent bone resorption which can be measured by standard techniques. Bone resorption is typically associated with a loss of estrogen. Bone resorption is typically associated with osteoporosis and is manifest by bone dissolution due to a loss of calcium.

By "uterine/breast sparing activity" is meant the prevention or reduction of the proliferation of tumorous cancer cells which can be measured by standard techniques.

Dihydronaphthalenes, benzothiophenes and other compounds described and suggested in Jones, U.S. Patent No. 4,418,068, issued November 29, 1983 (incorporated herein by reference), may be screened for keoxifene-like transcriptional profile. Other compounds that can be screened include compounds with a similar chemical structure to keoxifene or keoxifene-like analogs. Some of these compounds could be produced by making substitutions of 1-10 carbon long alkyl, alkenyl or similar-type chains in the nitrogen-containing ring of keoxifene. Other alterations could include altering the length or saturation characteristic of the alkyl chain (e.g., from 0-10 carbon atoms) that links the nitrogen-containing ring to the rest of the keoxifene compound. Other compounds that can be screened for a keoxifene-like profile include compounds with a chemical structure similar to tamoxifine or tamoxifine analogs. Those

skilled in the art will readily recognize other modifications and substitutions that can be made to compounds that can be screened for a keoxifene-like profile.

While steroids and steroid analogues may exemplify agents identified by the present invention, Applicant is particularly interested in the identification of agents of low molecular weight (less than 10,000 daltons, preferably less than 5,000, and most preferably less than 1,000) which can be readily formulated as useful therapeutic agents.

Such agents can then be screened to ensure that they are specific to tissues with pathological conditions related to an intracellular receptor with little or no effect on healthy tissues such that the agents can be used in a therapeutic or prophylactic manner. If such agents have some effect on healthy tissues they may still be useful in therapeutic treatment, particularly in those diseases which are life threatening.

Once isolated, a candidate agent can be put in pharmaceutically acceptable formulations, such as those described in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990), incorporated by reference herein, and used for specific treatment of diseases and pathological conditions with little or no effect on healthy tissues.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 shows schematic diagrams of ER-wt, ER-TAF1, ER-TAF2 (i.e., ER179C) and ER-Null mutants (Fig. 1A) and graphs indicating their transcription activation activities (Fig. 1B, C and D).

CV1, HepG2 and HS578T cells were transiently co-transfected with increasing concentrations of the

different receptor expression vectors as indicated, together with 9.5 μg/ml of ERE-tk-LUC reporter plasmid, and 5 μg/ml of pRSV-β-gal expression vector as an internal control for transfection efficiency. Carrier DNA (pGEM4) was added to adjust the total amount of DNA to 20 μg/ml (see below).

Cultures were treated with or without 10^{-7}M 17-β-estradiol (E_2) as indicated for 36 hours and assayed for β-galactosidase and luciferase activity. LUC activity is normalized for β-gal activity. The relative luciferase activity is calculated by dividing the normalized luciferase value at a given point by that obtained in the absence of a transfected receptor or ligand. A single experiment representative of four independent experiments is detailed above. Data shown indicate the mean ± SE(m) of triplicate estimations.

Fig. 2 shows schematic diagrams of ER-wt, ERN282G and ER-TAF2 (i.e., ER179C) mutants (Fig. 2A) and graphs indicating their transcription activation activities (Fig. 2B, C and D).

CV1, HepG2 and HS578T cells were transiently co-transfected with increasing concentrations of different receptor expression vectors as indicated, and assays were conducted as described in Fig. 1.

Fig. 3 shows diagrams indicating transcription activities of TAF1 and TAF2 on the human C3 gene promoter in different cells.

In Fig. 3A, 3B and 3C, CV1, HepG2 and HS578T cells were transiently co-transfected with 0.5 μg of the indicated receptor expression vector, 9.5 μg of C3-LUC reporter plasmid, 5 μg pRSV-β-gal and carrier DNA to a total amount of 20 μg DNA. In addition, a minus receptor control was included.

Cultures were treated with or without 10^{-7} M 17-β-estradiol (E_2) for 36 hours, and assayed for luciferase activity. The data shown are representative curves of experiments that have been repeated 6 times

with similar results. The curves represent averages of quadruplicate data points averaged and normalized for transfection efficiency by simultaneous estimation of pRSV-β-gal transcriptional activity.

Fig. 4 shows graphs indicating transcription activities of ER-TAF1 and ER-TAF2(i.e., ER179C) on different promoter constructs.

In Fig. 4A and 4B, CV1 and HepG2 cells were transiently co-transfected with 0.5 μg of the indicated receptor expression vector, 9.5 μg of pA_2-LUC reporter plasmid, 5 μg pRSV-β-gal and carrier DNA to a total amount of 20 μg. In Fig. 4C and D, CV1 and HepG2 cells were co-transfected as described above, using the pEREMLT-LUC reporter.

Cultures were treated with or without 10^{-7} M β-estradiol (E_2) for 36 hours and assayed for luciferase activity. Data presentation is described in Fig. 1.

Fig. 5 shows graphs indicating activation of ER-TAF1 and ER-TAF2 (i.e., ER179C) by triphenylethylene-derived estrogen partial agonists. ER represents ER-wt, ER^m represents ER-TAF1, TAF2 represents ER-TAF2 (i.e., ER-179C,) and $TAF2^m$ represents ER-Null.

HepG2 cells were co-transfected with 0.5 μg of the indicated receptor expression vectors, 9.5 μg of C3-LUC reporter, 5 μg of pRSV-β-gal and carrier DNA to a total amount of 20 μg.

Cultures were treated with 10^{-7}M of 17-β-estradiol (i.e., E_2, Fig. 5A), Tamoxifen (Fig. 5B), 4-hydroxy-Tamoxifen (Fig. 5C), Nafoxidine (Fig. 5D) or Clomiphene (Fig. 5E) for 36 hours and assayed for luciferase activity. Data presentation is described in Fig. 1.

Fig. 6 shows diagrams indicating displacement of estradiol binding to ER-wt and ER-TAF1 proteins by estrogen agonists.

Yeast cytosols prepared from cell expressing ER-wt or ER-TAF1 were incubated overnight at 4°C with 5 nM of ^{3}H-17-β-estradiol alone or in the presence of the

indicated concentrations of the different estrogen agonists. Ligand binding was determined by scintillation counting following separation of bound and free ligand using hydroxylapatite.

Fig. 7 is a schematic model showing TAF1 and TAF2 as functionally dependent activators of transcription.

This schematic outlines a hypothesis for the promoter and cell specificity of the individual transactivators of the estrogen receptor. Interaction of the receptor with ligand initiates a cascade of events which exposes the receptor DNA binding region (DBD) and promotes association of ER with DNA. Only "estrogenic compounds" are capable of functionally activating TAF2 region of the receptor. Upon activation (B), the TAF2 region of the receptor interacts with a transcriptional repressor (I), displacing it or altering its structure (C) to permit the TAF1 activation sequence access to the general transcription apparatus (GTA). In certain cells and on certain promoters, TAF2 function of the receptor can be supplied by other transcription factors, allowing TAF1 region of the receptor to function independently of TAF2. Therefore, binding of the receptor to DNA is synonymous with transactivation and can be accomplished by both estrogen agonists, as well as antagonists that permit delivery of the receptor to DNA. In this model, the partial agonist activity of the triphenylethylene-derived estrogen agonists depends on the conformation induced by the ligand and the effect that this conformation has on the presentation of TAF1 to the transcription apparatus.

Fig. 8 shows diagrams indicating that the partial agonist activities of the triphenylethylene derived antiestrogens depends on TAF1 function.

HepG2 cells were cotransfected with 0.5 μg of the indicated receptor expression vectors, 9.5 μg of C3-LUC reporter, 5 μg of pRSV-β-gal and pGem4 as carrier DNA to a total amount of 20 μg.

Cultures were treated with various concentrations of 17-β-estradiol (Fig. 8A), Clomiphene (Fig. 8B), Nafoxidine (Fig. 8C), 4-OH-Tamoxifen (Fig. 8D) and keoxifene (Fig. 8E) for 36 hours and assayed for luciferase activity. The data for panel E was obtained relative to a different estradiol control than the other panels. Thus, the peak in panel E appears approximately five times higher than it would if the data had been obtained relative to the same estradiol control that was used in panel A. The relative luciferase activity was calculated as described for Fig. 1. A single experiment representative of 6 independent experiments is detailed. The data shown indicate the mean $\pm$ SE(m) of triplicate estimations.

Fig. 9 is a diagram showing the effect of keoxifene (keox) on MCF-7 cell proliferation. The activity of estrogen in this assay is maximum at 10^{-10} M, and induces MCF-7 cell proliferation to 1500% of the control.

Fig. 10 is a diagram showing the structure of pC3-LUC plasmid.

Fig. 11 is a diagram showing functional domains of intracellular receptors.

Fig. 12 shows diagrams indicating schematic structure organization of PRB, PR-TAF1 (i.e., $PRB_{(E907A,E911A)}$), PR-TAF2 (i.e., PRA) and PR-Null (i.e., $PRA_{(E907A,E911A)}$).

Fig. 13 shows diagrams indicating activity of PR-TAF1 on MMTV promoter.

Plasmid phPRB or $phPRB_{(E907A,E911A)}$ was transfected into MCF-10 cells (A) or CV-1 cells (B) together with an MMTV-LUC reporter plasmid (10 μg/ml) and pCH110 (5 μg/ml) as an internal control. The amount of expression vector was chosen to permit maximal transcriptional activation in each cell line examined. The transfected cells were incubated for 40 h with increasing concentrations of progesterone as indicated, and assayed

for luciferase and β-galactoside activities. The data are presented as normalized luciferase (LUC) units.

Normalization was calculated by dividing the raw luciferase activity (relative light units x 10^4) for each point by the β-galactosidase activity (A415 x 10^5)/time in minutes) at that point. The data shown represent the mean values +/- the standard errors of the means of 12 replicates.

Fig. 14 shows graphs indicating activity of PR-TAF2 on TAT promoter in HeLa cells.

HeLa cells were transiently co-transfected with increasing concentrations of the hPR expression plasmid phPRB or phPRA (A), or phPRA or phPRA$_{(E907A,E911A)}$ (i.e., PR-TAF1) (B) together with a TAT-LUC reporter plasmid (10 μg/ml) and pCH110 (5 μg/ml) as an internal control. The transfected cells were incubated with or without 10^{-7} M progesterone as indicated for 40 h and assayed for luciferase (LUC) units calculated as for Fig. 13.

Fig. 15 shows graphs indicating activity of PR-TAF2 on MMTV promoter in HepG2 cells.

HepG2 cells were transiently co-transfected with increasing concentrations of the hPR expression plasmid phPRB or phPRA (A), or phPRA or phPRA $_{(E907A,E911A)}$ (i.e., PR-TAF1) (B) together with an MMTV-LUC reporter plasmid (10 μg/ml) and pCH110 (5 μg/ml) as an internal control. The transfected cells were incubated with or without 10^{-7} M progesterone as indicated for 40 h and assayed for luciferase and β-galactosidase activities. The data are presented as normalized luciferase (LUC) units calculated as for Fig. 13.

Fig. 16 (A-D) are diagrams showing saturation analysis of the binding of [^{3}H] progesterone to PRA and PRB mutants.

Aliquots of *in vitro* expressed receptor proteins PRB-wt (A), PRB$_{(E907A,E911A)}$ (B), PRA-wt (C) and PRA$_{(E907A,E911A)}$ (D) were incubated for 18 h at 4°C with increasing concentrations of [^{3}H] progesterone. Bound and free

ligand were separated using the dextran coated charcoal method. Each point represents duplicate determinations. Scatchard analysis of the saturation data are shown for each receptor protein in the inserts.

Fig. 17 is a graph showing the agonist activity of DHT on AR and the antagonist activity of 2-OH Fluramide on AR.

Fig. 18 is a graph showing an AR-TAF1 specific assay for screening AR-TAF1 agonists.

Figure 19 shows the structure organization of GR-wt, GR-TAF1, GR-TAF2, GR-N-del and GR-Null.

Fig. 20 shows the organization structures of GR-Gal4 constructs.

Fig. 21 is a graph showing the effect of mutations on TAF2 transactivation function.

Gal4 DNA binding domain fused to the ligand binding domain of either wt GR (Gal-G) or mutant galG. The number following Gal-G indicates the amino acid(s) that was mutated to alanine. These constructs were transfected into CV-1 cells. Dexamethasone was added to the cell culture. Each mutation resulted in a drastic reduction of the luciferase gene activation by dexamethasone.

Fig. 22 is a graph showing activation profiles obtained with different promoters.

Wild type and mutant GRs were co-transfected in CV1 cells with luciferase reporter genes driven by either MMTV, C3 or TAT3 promoters. Gene activation is measured after 24 hours of dexamethasone treatment.

Description of the Preferred Embodiments

The methods discussed briefly above are useful for identifying agonists or antagonists of various intracellular receptors, including, but not limited to, ER, GR, AR and PR.

ER, GR, AR and PR are members of the nuclear receptor super-family, a class of transcription factors

whose functions are regulated by steroids, vitamins or thyroid hormone (Beato, Cell 56:335, 1989). This family of regulatory proteins share common mechanistic features in that they are transcriptionally inactive within the cell until exposed to hormone. Occupancy by hormone results in transformation of the receptor to an activated state, thus allowing it to productively interact with specific DNA sequences in the regulatory regions of target genes. The resultant positive or negative effects of the bound receptor on specific gene transcription are cell-type and promoter-context dependent. Nonetheless, the relative effect may be measured in any particular cell/promoter construct. Thus, the desired effect may be observed in a wide variety of constructs.

The following are specific examples of this invention. These examples make use of the estrogen receptor, androgen receptor, progesterone receptor and glucocorticoid receptor, but are not limiting in the invention. Those in the art will recognize that other equivalent receptors, cells and promoters can be readily used in equivalent procedures within the scope of the claims.

I. Estrogen Receptor

The cDNA for ER has been cloned and used to reconstitute estrogen responsive transcription units in heterologous mammalian cells (Kumar et al., EMBO J. 5:2231, 1986, and Green et al., 231 Science 1150, 1986). This has enabled a detailed examination of the functional domains within the protein (Kumar et al., Cell 51:941, 1987). A functional examination of the domains of ER in several systems has revealed the likely structural features within the receptor which may interface with critical cellular components to generate a variety of hormone responsive endpoints (Danielian et al., EMBO J. 11:1025, 1992).

Two distinct transactivation domains have been defined, a sequence at the amino terminus of the receptor, termed TAF1, and a sequence confined to the carboxyl 60 amino acids, termed TAF2 (Danielian et al., supra; Berry et al., EMBO J. 9:2811, 1990; and Tasset et al., Cell 62:1177, 1990), all hereby incorporated by reference herein. Recently, investigators involved in intracellular receptor research have favored referring to the TAF domains, as AF domains (e.g. Cavailles et al., J. of Cellular Bio., 341, 1994) to avoid confusion with the discovery and cloning of TATA-binding protein associated factors (Dynlacht et al., 66 Cell, 563, 1991).

Pierre Chambon and his group (Kumar et al., Cell 51:941, 1987) have used truncated estrogen-receptor-encoding genes to study properties of the estrogen receptor and its alleged cell-type and promoter-context dependent activity. These truncated genes express ERs lacking all or a portion of two domains termed TAF1 and TAF2. These domains are thought to be regulated by estrogen and then cause promoter activation.

Tora et al., Cell 59:477 (1989), analyzed truncated mutants of human estrogen receptor, and described TAF1 and TAF2 as two transcriptional activation functions in the receptor. These activators are said to exhibit cell-type specificity and promoter-context dependency. The authors indicate that TAF2 acts synergistically with upstream elements.

Meyer et al., Cell 57:433 (1989), describe inhibition of transcription stimulation by the progesterone receptor by co-expression of the estrogen receptor. The authors propose that the observations reflect competition by the receptors for a limiting transcription factor.

Berry et al., EMBO Journal 9:2811 (1990), describe promoter-specific and cell-specific effects of an agonist on estrogen-responsive genes. Truncated and

chimeric estrogen receptors were used which contained TAF1 and/or TAF2 regions from the same or different sources.

Tassett et al., Cell 62:177 (1990), describe interaction of TAF1 and TAF2 regions, and competition (squelching) for limiting factors, by comparing relative activities of TAF regions and competitor constructs.

Fawell et al., Cell 60:953 (1990), describe estrogen-receptor dimerization and its alteration by mutations in the molecule.

Metzger et al., Nucleic Acids Research 20:2813 (1992), describe alleged promoter- and cell-specificity of TAF1 and TAF2 regions in the yeast *Saccharomyces cerevisiae*. Truncated receptors, or receptors having regions deleted from them, were used in the analyses.

Danielian et al., EMBO Journal 11:1025 (1992), describe conserved regions in the estrogen receptor and state that:

> Activities of TAF1 and TAF2 vary depending upon the responsive promoter and cell type and, in some cases, both are required for full transcriptional stimulation.

The authors identify amino acids near the C-terminus of the mouse estrogen and glucocorticoid receptors which are said to be essential for hormone dependent stimulation of transcription. Point mutations were introduced either into the full-length receptor, or into an internal deletion mutant which lacked the TAF1 region, to allow the authors to determine the effects of mutations upon TAF2 activity in the absence or presence of TAF1.

It appears that alterations in charged residues of the amino terminal portion of the hormone binding domain can result in increases or decreases in ER transcriptional activity with no change in receptor affinity of cognate hormone. Therefore, the regions around residue 530 (Danielian et al., supra,) and the

region around cysteine 381 (Pakdel et al., Mol Endocrinol, 7:1408, 1993) may in themselves constitute AF subdomains within TAF-2. It follows then, that changes in such domains, such as the region around cysteine 381, could result in mutant receptors which could discriminate between estrogen and antiestrogen ligands, paralleling the results obtained as detailed herein. Furthermore the analogous situation could exist for discreet residues in the TAF-1 region of ER.

The following experiments characterize the dependence of ER-TAF1 and ER-TAF2 activities on cell-context and promoter-context in mammalian cells, and the role of agonist or antagonist in the manifestation of these differences. Some of these experiments are described in Tzukerman et al., Mol. Endocrin 8:21, 1994, hereby incorporated by reference herein.

Receptor constructs

cDNA sequences encoding the ER-wt and a TAF1 - deleted receptor derivative were excised from the plasmids YEpwtER and YEpER179C, respectively, using BfrI and SacI. The DNA encoding the TAF1 receptor derivative was excised from the plasmid YePERN282G using BfrI and KpnI. Construction of the vectors YEpwtER, YEpER179C and YEpERN282G, have been described previously (Pham et al., Mol. Endo. 6:1043, 1992). The excised DNA was treated with T4 DNA polymerase (Boehringer Mannheim Co.) and ligated into the unique EcoRV site within the mammalian expression vector pRST7 (Berger et al., J. Steroid Biochem. Mol. Biol. 41:733, 1992).

The wild type estrogen receptor cDNA (ER-wt) was cloned into pGEM-11Zf(+) (Promega, Wisconsin). Specific mutations were introduced into the hormone binding domain of the receptor by substituting alanine for amino acids located at positions 538, 542, and 545, using site directed mutagenesis (Kunkel et al., 154 Methods in Enzymology 367, 1987), creating the plasmid pGERm. The

mutated hormone binding domains were introduced into ER-wt and ER179C by exchanging the BglII-SacI C-terminal fragment of this vector with the analogous mutated fragment from pGERm.

Reporter plasmids

The reporter ERE-tk-LUC contains a single copy of the vitellogenin ERE upstream of the herpes simplex thymidine kinase promoter sequences linked to luciferase (LUC).

The C3-LUC reporter contains 1.8 kb of the human C3 gene promoter (-1807 to +58) (Vik et al., Biochemistry 30:1080, 1991). Unique restriction sites Xho1 and BamH1 were introduced into the C3 promoter, the DNA was then cloned into the cognate sites of the vector p1-LUC (Berger et al., J. Steroid Biochem. Mol. Biol. 41:733, 1992), where a polyclonal site has been inserted into the MMTV-LUC vector (see Fig. 10). Those in the art can readily construct equivalent vectors.

pA2-LUC contains a 835 bp fragment (-821 to +14) of the *Xenopus* vitellogenin A2 gene promoter (Vik et al., Biochemistry 30:1080, 1991).

pEREMLT-LUC contains a single ERE upstream the adenovirus major late promoter sequences (-44 to +11) (Hu and Manly, Proc. Natl. Acad. Sci. USA 78:820, 1981).

Cell cultures

CV1 and HS578T cells were routinely maintained in Dulbecco's modified Eagle's medium - DMEM (Biowittaker, Maryland) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Utah). HepG2 cells were maintained in Minimal Essential Medium Eagle's - MEM (Biowittaker, Maryland) containing 10% FCS.

Transient transfection assay

Cells were seeded 24 hours prior to transfection in flat-bottom 96-well tissue culture plates ($5x10^3$

cells/well), in phenol red-free DMEM containing 10% FBS. DNA was introduced into cells using calcium phosphate precipitation. Plasmid DNA was diluted in 1 ml of 1 mM Tris, pH 7.4, 0.1 mM EDTA, 0.25 M $CaCl_2$. DNA solution was added dropwise with vortexing into an equal volume of 2X HBS pH 6.9 (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4) and precipitates were allowed to form for 20 minutes. Transfections (11 μl of DNA mix/well) were performed on a Biomek 1000 Automated Laboratory Workstation (Beckman, California). Cells were transfected for 6 hours and then washed with phosphate-buffered saline (PBS) to remove the precipitate. Cells were incubated for an additional 36 hours in phenol red-free medium containing 10% charcoal-treated FBS, with or without hormones as indicated in the text. Cell extracts were prepared as described by Berger et al., J. Steroid Biochem. Mol. Biol. 41:733, 1992, and assayed for luciferase and β-galactosidase activities. All determinations were performed in triplicate in at least two independent experiments, and were normalized for transfection efficiency by using expression of β-galactosidase as an internal control.

Preparation of yeast receptor proteins

Expression vectors producing ER-TAF1 were constructed by replacing the Bfr1-Mlu1 fragment of YEpE10 (Pham et al., 88 Proc. Natl. Acad. Sci. USA 3125, 1991) with the corresponding fragment of pRST7ER-TAF1. This vector and a vector producing wild type receptor (YEPE10) were transformed into the yeast strain BJ2168 as described by McDonnell et al., J. Steroid Biochem. Molec. Biol. 39:291, 1991. Individual transformants were picked and grown to an OD_{600}=1. Cultures were then induced with 100 μM $CuSO_4$, and 2 mM chloroquine for 16 hours at 30°C. Cells were then pelleted and washed with cold water. Cells were resuspended in 2-5X pellet volume of 10 mM Tris, 0.4 M KCl, 2 mM EDTA, 0.5 mM PMSF,

1 μg/ml aprotinin, 2 mM DTT, pH 7.6, and lysed by vortexing with 0.45-0.5 mm glass beads, intermittently with cooling on ice, until at least 90% of the cells were observed to be open. Extracts were centrifuged at 13,000xg and the supernatants were recovered. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

β-Estradiol binding competition assay

This assay was performed on a Biomek 1000 automated workstation (Beckman Instrument, Fullerton, CA). Ten-fold serial dilutions of the compounds to be tested were made in 10 mM Tris, 0.3 M KCl, 5 mM DTT, pH 7.6, and transferred to polystyrene tubes containing 100 μl at final concentrations of 10^{-4} M to 10^{-11} M diluted compounds, 5 mM ^{3}H-β-estradiol (Amersham, UK), and 22 μg total protein derived from strains producing ER or ER-TAF1. Following an overnight incubation at 4°C, 100 μl of a 6% hydroxylapatite slurry in 10 mM Tris, 5 mM DTT, pH 7.6 was added. The tubes were incubated for an additional 30 minutes at 4°C, mixing after the first 15 minutes. Hydroxylapatite pellets were washed 4X with 1 ml 1% Triton X-100 in 10 mM Tris, 5 mM DTT, pH 7.6. Finally, the hydroxylapatite pellets were resuspended in 800 μl of Ecoscint A scintillation fluid (National Diagnostics, Manville, NJ). Activity in each sample was measured using a LS6000IC scintillation counter (Beckman Instruments, Fullerton, CA).

Example 1: ER-TAF1 Transcription Activation Requires the Functional Context of TAF2

TAF1 and TAF2 functions have been defined as individual domains within the estrogen receptor that were capable of supporting transcription of an ER responsive promoter (Berry et al., 9 EMBO J. 2811, 1990, and Tasset et al., 62 Cell 1177, 1990). However, we were unable to show a distinct activity of the TAF1

sequence when analyzed in the context of the ERN282G deletion in the mammalian cells tested here. Therefore, we considered whether analysis of this transactivator outside the context of the full-length receptor may not reflect its true biological activity.

Danielian et al., supra, changed three amino acids between residues 535 and 550 in the carboxyl terminus of the mouse estrogen receptor which comprises transcriptional activity of TAF2 without diminishing the receptor's ability of binding both specific DNA and cognate ligand with wild type affinity, indicating that these changes did not lead to gross structural abnormalities in the protein.

We created similar amino acid changes in the carboxyl terminus of human ER at residues 538, 542 and 549 using site-directed mutagenesis (see the ER-TAF1 construct of Fig. 1). This triple mutation was also introduced into ER179C creating a null estrogen receptor (See the ER-Null construct of Figure 1). ER-Null and ER-TAF1 constructs allowed a specific determination of the effect of these mutations on TAF2 function. The transcriptional activities of these mutant ERs were assessed by transient transfection into CV-1 cells together with the ERE-tk-LUC reporter.

Mutation of these three amino acids provides but one example by which the context of a TAF region can be maintained while inactivating that region. Those in the art will recognize that equivalent mutations in the same or other amino acids can be readily made by standard techniques.

Introduction of the triple mutation into ER179C totally abolished TAF2 activity (Fig. 1B). Thus, we believed that introduction of this mutation into the wild-type ER would allow an examination of TAF1 activity in the full-length receptor context without interference from TAF2 activity. The ability to specifically mutate the TAF2 activator within the human estrogen receptor in

this manner is consistent with the results previously reported for the mouse ER, and indicates that equivalent mutations can be made in the other receptor TAF regions.

The full-length receptor containing the triple mutation (ER-TAF1) was subsequently used for analysis of TAF1 function in the context of the intact receptor. Constructs encoding wild type receptor, ER-TAF1, ER-TAF2 (i.e., ER179C) or ER-Null were transfected into CV-1, HepG2 or HS578T cells, together with the ERE-tk-LUC reporter. The results are shown in Fig. 1.

In all cell lines, the ER179C was transcriptionally active, as observed earlier (Figs. 1B, C & D), whereas the null receptor was inactive. Interestingly, in CV-1 cells, in the absence of a functional TAF2 activation sequence, the ER-TAF1 protein exhibited a significant transcriptional activity (Figs. 1B, C & D). Thus, the activity of the TAF1 activator when analyzed in the context of a full length receptor molecule, as observed here, was different from that when analyzed as a deletion mutant (ERN282G, Fig. 2). This suggests that TAF1 activity does not function independently, but rather requires additional carboxyl-terminal sequences for appropriate function.

Interestingly, increasing the concentration of transfected ER-TAF1 DNA did not result in a receptor dependent "squelching" of transcriptional activity. This observation implies that both TAF1 and TAF2 activators and possibly the context of the full-length receptor are required for this squelching function.

A comparison was made of the expression level of each of these receptors by transfecting the expression vectors into CV-1 cells and analyzing the hormone binding activities in the resulting cytosolic extracts (data not shown). The K_ds of the ER-wt, ER-TAF1 and ER179C were the same. The ER-wt and ER-TAF1 were synthesized in comparable levels as measured by hormone binding activity, whereas the amino-terminally deleted

ER179C and the null receptor were expressed at about 25% of ER-wt level. Since all the transcriptional responses detected with each receptor were maximal responses achievable, it is unlikely that receptor expression levels are a significant factor in the outcome of our experiments.

Example 2: Transcriptional Activation by TAF1 and TAF2 Truncated Receptors

Referring to Fig. 2, truncated forms of the human estrogen receptor were prepared which lacked either the TAF1 (ER179C, see Fig. 2A) or the TAF2 (ERN282G, see Fig. 2A) activation sequence. These constructs encode proteins structurally similar to those used previously in mammalian (Berry et al., 9 EMBO J. 2811, 1990) and yeast cells (Pham et al., 6 Mol. Endo. 1043, 1992).

The transcriptional activities of these ER derivatives were assessed using a reporter plasmid containing one copy of the vitellogenin estrogen response element (ERE) (Klein-Hitpass et al., 76 Cell 1053, 1986) inserted upstream of the thymidine kinase promoter (ERE-tk-LUC). The reporter plasmid and increasing concentrations of ER or mutant ER expression vectors were transiently transfected into the ER negative cell lines CV-1 (monkey kidney fibroblasts), HepG2 (human hepatocellular carcinoma) and HS578T (human breast cancer cells), and activity assessed as documented in Fig. 2B. All transfections were performed in the absence or in the presence of 17-β-estradiol at concentrations ranging from 10^{-5}M to 10^{-11}M. However, due to the number of data points obtained in this way (>2,500) only the activities obtained using 10^{-7}M 17-β-estradiol are presented since this is a concentration that elicited maximal transcriptional response in all cell lines examined.

The ER-wt was active in all cell lines. Using this protocol, we were unable to detect significant TAF1-

mediated transcriptional activity in either CV-1, HepG2, HS578T (Figs. 2B, C, D) or HeLa or U2OS cells (data not shown) when assayed in the context of the ERN282G deletion. In contrast, however, the TAF2 activation function (ER179C) exhibited substantial activity in these cells (Figs. 2B, C & D). The magnitude of the TAF2 transcriptional activity by ER179C was cell-type dependent. This isolated activator exhibited a lower efficacy relative to wild type receptor, even at DNA concentrations that produced saturating receptor levels. In HepG2 cells ER179C was about 35% as active as ER-wt (Fig. 2C), whereas in CV-1 and HS578T, the ER179C demonstrated 70% and 65% of ER-wt activity respectively (Figs. 2B & D). Transfection efficiency and recombinant expression levels were similar as estimated by indirect fluorescence microscopy and flow cytometric analysis (data not shown).

The results obtained in this analysis are consistent with the hypothesis that the TAF1 and TAF2 sequences represent functionally distinct transcriptional activators. A wild type receptor activity requires either both activator regions or an intact receptor context for an individual activator to exhibit maximal transcriptional activity.

In addition to the partial activities observed by the above ER-mutants, increasing concentrations of transfected ER-wt in CV-1 and HS578T cells led to a progressive decrease in hormone dependent transcriptional activity (Figs. 2B & D). This phenomenon has been observed by others and likely results from sequestration of limited transcription factors or targets by the over-expressed, hormone-activated receptor, such that activated receptor function is compromised (Tasset et al., 62 Cell 1177, 1990). This "squelching" or "transcriptional interference" supports the idea that ER requires additional, limiting cellular transcription factors for

appropriate function. The failure of the ER-wt to "squelch" in the HepG2 cell line (Fig. 2C) suggests either an increased abundance of a required co-factor, or the involvement of an additional cell specific component in this process.

Example 3: ER-TAF1, HepG2 Cell and C3 Promoter Constitute an Assay for an Agonist or Antagonist Acting Through TAF1

The above results using the ERE-tk-LUC reporter indicated that the TAF1 activator of the estrogen receptor functions, albeit weakly, in the absence of an intact TAF2 function. In addition, TAF1 activity appeared to be cell-type dependent.

We extended our studies to examine the efficacy of the individual activator functions on other estrogen responsive promoters. To this end, we chose the estrogen responsive C3 promoter in which a strong ERE has recently been identified (Zawaz, Gene Exp. 2:39, 1992; Vik et al. Biochemistry 30:1080, 1991).

The activities of the of ER-wt, ER-TAF1 and ER-TAF2 (i.e., ER179C) activators were evaluated on C3 promoter-directed transcription as depicted in Fig. 3. In HS578T cells, the C3 promoter can be activated equally well by either ER-wt, ER-TAF1 or ER179C (Fig. 3C). In HepG2 cells, however, the ER-TAF1 activator was as active in transcription as wild type ER, but the ER179C activator was silent (Fig. 3B).

These data suggest that, with respect to the C3 promoter, there is a strong cell-type bias in ER transactivator functions. In CV-1 cells it appears that the combination of the activation sequences is required for maximal activity (Fig. 3A). Cumulatively, these data suggest that the TAF1 and TAF2 activators within ER demonstrate a dependence upon cell-type and promoter, and furthermore, the dominant activator of ER-mediated regulation of C3 expression is TAF1.

The analysis of the relative contribution of the individual ER TAF domains in ER function was extended to include two additional promoters, namely the adenovirus major late promoter, containing an estrogen response element, and the vitellogenin promoter (Fig. 4). Both of these promoters were responsive to estrogen in the presence of ER-wt. However, unlike the C3 promoter the individual activation domains of ER were minimally active in both cell lines examined. This highlights further the promoter specificity of the estrogen receptor activation domains. Similar tests to those described above can be used to quickly identify useful promoter and cell combinations for use in assays for agonists discussed above (see also, Example 4, below).

<u>Example 4</u>: <u>Screening Agents for Partial Agonist Activity</u>

Certain triphenylethylene-derived estrogen receptor antagonists (<u>i.e.</u>, tamoxifen, nafoxidine) are reported to exhibit partial agonist activities. We tested whether these compounds preferentially activate either TAF1 or TAF2 transactivators.

A series of these compounds was evaluated in HepG2 cells using the ER-TAF-specific receptor derivatives and the C3 promoter. In this cellular and promoter context, ER179C was not activated by either estradiol or the partial estrogen agonists. On the C3 promoter, tamoxifen, 4-hydroxy-tamoxifen, nafoxidine and clomiphene were all potent activators of ER-wt mediated C3 gene transcription (Figs. 5B-E). However, none of these compounds were as effective as estrogen in this regard (Fig. 5A). In addition, estrogen was an efficient activator of the ER-TAF1, whereas the partial estrogen agonists were not as effective.

These data imply that even though TAF1 activity is necessary, it alone does not activate this promoter by triphenylethylene-derived antihormones, suggesting that

their mode of action may be mechanistically different from that of estrogen (Figs. 5A-F). The differences in hormonal responsiveness of these receptor derivatives do not relate to alterations in the affinity of the proteins for ligands. As shown in Figs. 6A and B, the affinity and specificity for ligands of the ER-wt and ER-TAF1 were indistinguishable. It is notable that not all the anti-hormones tested in this system have an identical transcriptional profile (Figs. 5B-E). The absolute efficacy for each of the anti-hormones is different, as is their ability to differentially activate TAF1, suggesting subtle mechanistic differences in the agonistic properties of these ligands.

These examples demonstrate specific point mutations in the human estrogen receptor affects ER-transcription activation function. Mutation of TAF2 in this manner is still compatible with wild-type binding affinities for estrogen, tamoxifen and 4-hydroxy-tamoxifen. Surprisingly, TAF1 retained considerable transcriptional activity despite TAF2 mutation. When we deleted the entire TAF2 sequence (ERN282G) we were unable to observe residual transcriptional activity of TAF1 in any cell line examined. This suggests that either TAF2 or the context of the full length receptor is required for full manifestation of TAF1 activity.

Berry et al. have observed that a construct identical to ERN282G was constitutively active in avian CEF cells (Berry et al., 9 EMBO J. 2811, 1990). This may suggest a difference in estrogen receptor function in mammalian and avian cells, and may not reflect basic differences between the two sets of results.

Using the modified receptors we were able to identify cell and promoter specific differences in the activity of ER-TAF1 and ER-TAF2 (i.e., ER179C). In studies which were controlled for expression level and transfection efficiency we saw that both activators displayed promoter and cell type specific differences in

their activity. The most striking example of this is the inability of ER179C to function well on any promoter in HepG2 cells. The ER-TAF1 activator, on the other hand, functions very well on the complex C3 promoter, but less well on the other promoters examined. The activity profiles of ER-TAF1 and ER179C are clearly distinct, suggesting dissimilar mechanisms of action. On the complex C3 promoter there is no apparent synergism between TAF1 and TAF2, whereas it clearly exists on other promoters. This suggests that on this promoter the ER activation domains may interact with differing transcription factors. The data obtained using the C3 promoter in HepG2 cells indicate that there is a transcription factor in these cells that can functionally replace TAF2, as TAF1 is as good a transcriptional activator as ER-wt. However, since TAF2 alone does not activate transcription, it suggests that no mimetic for TAF1 exists for transcription of this promoter in this particular cell line.

The dissimilar mechanism of action and the promoter and cell type specificity can be explained by a model in which TAF1 is the dominant transcriptional activator and TAF2 is a transcriptional facilitator (see model in Fig. 7). We suggest that the function of TAF2 is to "prepare" the transcription apparatus for TAF1 function. This "preparation" function could be the recruitment of basic transcription factors, alteration of chromatin structure or overcoming the effects of a transcriptional repressor. On the other hand, TAF2 could "prepare" the transcription apparatus for another transcriptional activator, but on its own would have little inherent transcriptional activity. In support of this hypothesis, the TAF2 activator is poorly active on minimal promoters.

This dependence on promoter complexity is also observed for ER-TAF1 activity. Additional evidence in support of the facilitator role of TAF2 is that in yeast

the TAF1 activator is inactive on a minimal promoter. However, a mutation of the SSN6 locus (a cellular repressor of transcription), Keleher et al., 68 Cell, 709, 1992, results in a 100-fold increase in ER-TAF1 activity (to a level comparable to ER-wt), whereas ER179C activity is not effected (McDonnell et al., 89 Proc. Natl. Acad. Sci. USA 10563, 1992). We suggest that this cellular mutation has the effect of mimicking the function of TAF2, and that in mammalian cells TAF2 has a similar role. In this system TAF1 is unable to access the transcription apparatus as a result of stearic hindrance by an inhibitor. Where TAF2 is available, then the inhibitor is displaced and TAF1 is able to interact with the transcriptional apparatus.

Example 5: Screening For and Use Of Compounds With Keoxifene-Like Transcriptional Profiles

In humans the tri-phenylethylene derived anti-estrogen keoxifene exhibits bone sparing activity while having no significant effects on uterine proliferation. In contrast, tamoxifen, a related anti-estrogen, is bone sparing but functions as a partial estrogen agonist in the uterus promoting an undesirable proliferative effect.

In order to determine whether the differences in the in vivo biological activity of tamoxifen and keoxifene could be reconciled by their differential ability to transcriptionally activate TAF1, these compounds were studied in HepG2 cells using the ER-TAF specific receptor derivatives on the C3 promoter. The results are shown in Figure 8D and 8E.

Keoxifene had a unique transcription profile in this promoter and cellular context. In particular, low concentrations of keoxifene stimulated ER transcriptional activity. At higher concentrations, keoxifene inhibited the basal transcriptional activity of ER and did not cause further transcriptional

activation (See Fig. 8E). On the ER-TAF1 construct, keoxifene demonstrated significant partial agonist activity inducing C3 promoter transcription 5-fold over background. The mechanism by which keoxifene manifests a transcriptional profile distinct from the related molecule tamoxifen is unclear. However, it is likely that these compounds induce subtle alterations in receptor structure which facilitate distinct interactions of the ER-TAFs with the general transcription apparatus.

The unique profile exhibited by keoxifene in this <u>in vitro</u> assay suggested that additional compounds displaying similar transcriptional profiles may also exhibit favorable bone protective/uterine sparing activities. To this end we have studied several compounds derived from tamoxifen using this assay system and have been able to split these compounds into three distinct groups based on their ability to modulate TAF1 activity. The first category contains compounds that resemble the activity of estrogen, the second group resembles the activity of tamoxifen and the third group profile similar to keoxifene.

When the keoxifene-like compounds are assayed in the ovariectomized rat model they are expected to be bone protective and to demonstrate no uterotrophic activity. Rats are given a dorsal ovariectomy as follows:

The animals are anesthetized with Ketamine:Xylazine and surgery is performed. Shave the central back of the anesthetized rat. Make a longitudinal incision in the skin parallel to the spine about 1 inch long. Spread the connective tissue away from the muscle layer with the tips of scissors. About 1 inch from the spinal column at the base of the rib cage, make a small cut (1/4") of the muscle with scissors. With small forceps, pull out ovarian fat. Ovary will be visible as a cluster-like structure, attached to the end of the uterine horn. Cut the connective tissue that holds them

together. Staunch any bleeding. Replace fat into body cavity. Repeat on opposite side. Clip skin together and use betadine on the incision to retard infection and reduce clip removal. The next day injections are begun. Injections are done subcutaneously in the hip daily (usually in the morning). Vehicle is 10% ethanol and all injection volumes are 300 μl. After 28 days of injections, the animals are sacrificed under anesthetic by cervical dislocation and the body and wet uterine weights are determined. The hindlimbs are taken for histology and histomorphometry.

The transcriptional profile exhibited by keoxifene in vitro is predictive of agents demonstrating bone selective estrogenic activity. Thus, the use of these ER-TAF constructs examined on this promoter and cellular context provides a useful screen for compounds useful for the treatment of osteoporosis.

In in vivo studies, rats are subjected to sham or authentic ovariectomy and allowed to recover for 5 days. Rats (4-6 per group) are then injected subcutaneously with vehicle or vehicle containing estrogen, keoxifene, or a test compound daily for periods up to 28 days. Animals are sacrificed, weighed, and evaluated for uterine wet weight, total serum cholesterol, and bone mineral density. Established methods are utilized with the exception that bone mineral density of the distal femural metaphysis is determined utilizing an Hologic mineral densitometer. Bone marrow from test animals is evaluated for osteoclastic potential in the coculture assay with primary osteoblasts. Bone marrow is combined with primary osteoblasts in the presence of 1,25-dihydroxyvitamin D3 and parathyroid hormone for 8 days and scored for the number of tartrate acid phosphatase resistant multinucleated cells (TRAP + MNC). The number of TRAP + MNC in the sham operated animals is set at 100%. Tartrate resistant acid phosphatase positive,

multinucleated cells are scored by standard methods as nascent osteoclasts.

The *in vitro* effects of compounds on MCF-7 breast cell proliferation can also be studied. The partial agonist activities of estrogen, keoxifene, and the test compound on MCF-7 human breast carcinoma cells, is assessed by treating the cells for 7 days in the absence or presence of increasing concentrations of compound. Cells are treated at day 0 and day 4 with compound. Triplicate wells are evaluated for cell number at termination of the experiment on day 7. The activity of estrogen in this assay is expected to be maximum at 10^{-10}M, and induce proliferation to 1500% of control.

The *in vitro* profiles of keoxifene and the test compounds could then be determined. The activities of increasing concentrations of compound on ER, ER-TAF1, and ER-null receptor transactivation of the C3 promoter in HepG2 cells is determined by standard methods as described in this document. Thus, compounds that exhibit both bone protecting and uterine/breast sparing activity at a given concentration can be identified. Higher potency compounds than keoxifene are preferred. By "potency" is meant the amount of a compound required to produce the desired effect. Thus, high potency compounds will bind to the receptor with greater affinity than keoxifene. High potency compounds produce maximal effect at minimal dosages. As illustrated in Fig. 8, compounds with high potency have peaks further to the right than do compounds of lower potency.

ER agonists and their type can be quickly identified in the above systems. Specifically, the experiment described in Example 4, and illustrated in Fig. 5, is useful for identifying an agonist and then defining its type of activity. For example, the use of a wild type receptor (ER-wt) in this assay will indicate whether a test compound is an ER agonist. The use of a mutated

receptor with full functional context (ERm) in the assay will indicate the type of agonist, i.e., what level of activity is observed. Examples of the range of results expected with various test compounds are shown in Fig. 5, and discussed in Example 4. Using such assays, one can readily screen for desired agonist activity, e.g., agonists active only at TAF1 regions which mimic the activity of estrogen and are useful for treatment of osteoporosis.

II. Progesterone Receptor

The steroid hormone progesterone is involved in the regulation of growth and development of mammary gland and uterus. Synthetic progestins and antiprogestins have been used or are in human clinical trials in treatment of endometrial and breast cancer, as combination oral contraceptive agents, and as adjuncts to estrogen in hormonal replacement therapy.

The effects of progesterone are mediated by progesterone receptors. Upon binding to their hormonal ligands, the activated receptors bind with high affinity to specific DNA binding sites and activate transcription of the cis-linked genes.

Gronemeyer et al. (1987) EMBO J. 6:3985-3994, cloned and sequenced chicken PR and described that both the N-terminal A/B region and C-terminal hormone binding domain are required for transactivation function on MMTV promoter in HeLa cells.

Carson et al. (1987) Mo. Endo. 1:791-801, cloned and sequenced chicken PR, and conducted analysis to define the functional domains of the receptor. The authors state that a transcription activation function domain resides outside the hormone binding domain and that the C-terminal region may function as a "repressor".

Tora et al. (1988) Nature 333:185-188, reported on the functional difference between chicken PRA and PRB in the transactivation of target genes.

Meyer et al. (1990) EMBO J 9:3923-3932, described the presence of two transcription activation function (TAFs) located in the N-terminal region A/B (TAF1) and the hormone binding region (TAF2) of the human PR. The authors described the agonistic activities of RU486.

Meyer et al. (1992) J Biol Chem 267:10882-10887, state that a region specific for PRB is required for TAF1 function.

Two isoforms of human progesterone receptor, sized 94kD and 120 kD, have been observed in human breast cancer T47D cells and primary human endometrial carcinoma. In T47D cells, two promoters in a gene give rise to two distinct classes of human progesterone receptor mRNAs, one of which codes for hPRB while the other one encodes hPRA. The two isoforms differ only in their N-terminal sequences. Form A lacks the amino terminal 164 amino acids present in form B.

The human progesterone receptor A and B isoforms (hPAR and hPRB) differentially activate transcription of progesterone-responsive genes (Vegeto et al., Mol. Endo. 7:1244-1255, 1993; Wen et al., Mol. Cell. Biol. 14:8356-8364, 1994, all incorporated by reference herein). Using site-directed mutagenesis we were able to show that hPRA has a functionally inactive TAF1. TAF2 of hPRA is the sole transcription activator of this receptor form. However, TAF2 is a weak transcription activator and functions only in some promoter and cellular contexts.

TAF1 of hPRB functions synergistically with TAF2 of hPRB in certain promoter and cellular contexts. TAF1 of hPRB also functions independently in some promoter and cellular contexts.

Reagents

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Indianapolis, Ind.), New England Biolabs (Beverly,Mass.), or Stratagene (San

Diego, Calif.). PCR reagents were obtained from Persin-Elmer Cetus (Norwalk, Conn.). Progesterone, dexamethasone, and 17-β-estradiol were purchased from Sigma (St. Louis, Mo.). [1,2-^{3}H]progesterone was purchased from Amersham (Arlington Heights, Ill.).

Receptor and reporter constructs

Construction of the mammalian expression plasmids phPRB, phPRA and pRShGR has been described elsewhere (Vegeto et al. (1993) Mol. Endocrin. 7:1244-1255), as has the construction of PRE$_2$-TK-LUC,TAT-LUC, and ERE-TK-LUC reporters (Berger et al., (1992) Cell 70:251-265,Tzukerman et al., (1994) Mol. Endocrinol. 8:21-30,Vegeto et al. (1993) Mol. Endocrin. 7:1244-1255).

Plasmid MMTV-ERE-LUC was constructed as follows. Plasmid ΔMTV-LUC containing a deletion of the sequences from +190 to -88 was obtained from Ron Evans (Salk Institute, San Diego, Calif.). This plasmid was digested with *Hin*dIII to remove the glucocorticoid response elements, and five copies of a 33-bp oligonucleotide containing the consensus vitellogenin A2 estrogen response element were inserted. The sequence of the oligonucleotide used was 5'AATTAAAGTCAGGTCACAGTGACCTGATCAAA3'.

Glutamic acid residues at positions 907 and 911 were substituted with alanines by using PCR primers containing two base changes (underlined) as indicated: 5'CCAG<u>C</u>AATGATGTCTG<u>C</u>AGTTATTGC3' and 5'GCAATAACT<u>G</u>CAGACATCATT<u>G</u>CTGG3' (National Biosciences Inc., Plymouth, Minn.) to prepare the E907A and E911A mutations. The *Eco*NI-*Kpn*I fragment of DNA containing the mutated receptor sequences was subcloned into plasmids phPR-B and phPR-A and sequenced to confirm the mutations.

Cell lines

The human mammary epithelial cell line MCF-10 was obtained originally from Samuel Brooks (Michigan Cancer Foundation). This cell line was routinely maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (Biofluids, Rockville, Md.) and Ham's F12 medium (Biofluids) with 20 ng of epidermal growth factor (Sigma) per ml, 100 ng of cholera toxin (Sigma) per ml, 0.01 μg of insulin (Biofluids) per ml, 500 ng of hydrocortisone (Sigma) per ml, and 5% horse serum (Biofluids). The human breast adenocarcinoma cell line MCF-7 was obtained from Marc E. Lippman (Vincent T. Lombardi Cancer Center, Georgetown University) and maintained in Iscove's modified Eagle's medium (Biofluids) with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah). Monkey kidney CV-1 fibroblasts were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Assays

Cotransfection assays were conducted as follows. Cells were plated in 12- or 96-well tissue culture plates the day before transfection. DNA was introduced into cells by the calcium phosphate coprecipitation method (Berger et al., (1992) Mol. Biol. 41:733-738). For each transfection reaction, 20 μg of DNA per ml of transfection buffer was used. For the 96-well plate experiments, transfections were performed with a Biomek 1000 automated laboratory workstation (Beckman, Fullerton, Calif.). Cells were incubated with the precipitate for 6 h, then washed with phosphate-buffered saline, and incubated for 40 h with or without hormones as indicated in the text. Cell extracts were prepared as previously described (Berger et al., (1992) Mol. Biol. 41:733-738) and assayed for luciferase and β-galactosidase activities.

Hormone binding assay

Hormone binding assays were conducted as follows. The wild-type PR and mutant receptor proteins were produced by in vitro translation of mRNA synthesized by using wild-type and mutant PR templates. The binding assay buffer constituted of 10% glycerol, 10mM Tris, 2 mM dithiothreitol, 2 mM 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, and 1.5 mM EDTA (pH 7.5). The binding assays were performed in a 500-μl volume containing 10 μl of reticulocyte lysate (containing PR) and various concentrations of [^{3}H]progesterone in the absence or presence of 10 μM progesterone. Incubations were carried out at 4°C for 16 h. At the end of the incubation period, bound and unbound progesterone were separated by using dextrancoated charcoal. The supernatants containing bound progesterone were drawn off, and the radioactivity retained was estimated by liquid scintillation counting. Data were analyzed by the method of Rosenthal, H.A. (1967) Anal. Biochem. 64:1393-1401. For competition binding assays, a similar protocol was used except that a fixed concentration of [^{3}H]progesterone was added to extracts in the presence or absence of competing ligands (1nM to 10μM). After correcting for nonspecific binding, 50% inhibitory concentration values were determined graphically from a log-logit plot of the data. K_is were determined from the calculated 50% inhibitory concentration values by using the Cheng-Prusoff equation (Cheng et al., (1973) Biochem. Pharmacol. 22:3099-3108).

A PR-TAF1 construct, phPRB$_{(E907A,E911A)}$, was transfected into MCF-10 cells together with an MMTV-LUC reporter plasmid (see Figs. 12 and 13). In MCF-10 cells, phPRB$_{(E907A,E911A)}$ functioned as a hormone-dependent activator of MMTV gene transcription in the presence of progesterone (Fig. 13A). In contrast, this mutant

receptor was unable to activate MMTV gene transcription in CV-1 cells (Fig. 13B).

Therefore, MCF-10 cells, phPRB$_{(E907A,E911A)}$ and MMTV promoter constitute an assay for an agonist or antagonist which acts through the TAF1 region of PR. With this assay, we were able to demonstrate that the partial agonist activity of some antiprogestins is mediated by the TAF1 region of hPRB.

A PR-TAF2 selective assay was also constructed. A PR-TAF1 construct, phPRA, was transfected into HeLa cells together with a TAT-LUC reporter plasmid (see Figs. 12 and 13). The construction of plasmids, mutagenesis, cell culture, cotransfection assays and hormone binding assays were conducted as described in Wen et al. supra.

In HeLa cells, phPRA functioned as a hormone-dependent activator of TAT gene transcription in the presence of progesterone (Fig. 14). In contrast, this mutant receptor was unable to activate MMTV gene transcription in HepG2 cells (Fig. 15). Therefore, HeLa cells, phPRA and TAT promoter constitute an assay for an agonist or antagonist which acts through the TAF2 region of PR.

Depending on the expression ration of hPRA and hPRB and the particular target tissues or cells, PR agonists can activate the transcription of different sets of genes. PR antagonists can have different effects of target gene transcription depending on cellular environments. Partial agonists or antagonists of PR screened by the above described assays may be used as oral contraception, in hormone replacement therapy, to treat endometriosis, fibroids, endometrial cancer and breast cancer with little or no side effects.

III. Androgen Receptor

The steroid hormones testosterone and its active metabolite dihydrotestosterone (DHT) have multiple

effects in the body, including fusion of the labioscrotal fold during embryogenesis, induction of male differentiation of the wolffian ducts, growth of the male urogenital tract, induction of spermatogenesis, growth of beard and body hair, retention of nitrogen, temporal regression of scalp hair, hyperplasia of the sebaceous gland with increased sebum production, development of prostatic hyperplasia in aging males, secretion of ejaculate, and virilization of the hypothalamus. These diverse effects are modulated through the action of the androgen receptor AR.

The human AR cloned by Lubahn et al. Mol. Endo. 2:1265-1275, 1988, is a member of the superfamily of ligand inducible nuclear receptors. AR has high homology with PR, MR and GR (Evans R.M. Science 240:889-895, 1988).

Chang et al. Proc. Natl. Acad. Sci. 85:7211-7215, 1988, described the cloning of AR and androgen binding specificity.

Jenster et al. Mol. End. 5:1394-1404, 1991, described a series of deletion mutants of AR that define steroid binding domain, regions of the N terminal region required for transactivation and regions required for nuclear targeting.

Palvimo et al. Mol. Endo. 7:1399-1407, 1993, described a region of the N terminal domain that is required for transactivation.

When testosterone or DHT binds to hAR, hAR binds to HREs in target genes and modulates transcription of those genes. DHT causes a dose-dependent enhancement of luciferase activity from cells cotransfected with a hAR plasmid and a reporter plasmid containing an androgen response element (Figure 17). The activity induced by DHT can be reversed by the nonsteroidal AR antagonist, 2-hydroxy flutamide.

Receptor Constructs

Four receptor constructs were generated to establish an assay to screen for AR TAF region partial agonists and antagonists. These constructs are analogous to the mutant forms of the estrogen receptor described above.

cDNA sequence encoding the AR-wt was excised from plasmid hARpGEM3 as a 3.6Kb BglII/BamH1 fragment and ligated into unique BamH1 site of the mammalian expression base vector pRS (Berger et al., 41 J. Steroid Biochem. Mol. Biol. 733,1992). The resulting plasmid was named pRShAR. pRShAR-wt contains the coding sequence of the full length human AR cloned into the expression vector pRS (AR-wt plasmid).

The AR-TAF1 plasmid was generated by cloning a BamH1/Kpn1 fragment of the hAR into the plasmid pALTER using an *in vitro* mutagenesis system by Promega. Specific mutations were introduced into the hormone binding domain of the receptor by substituting alanine for amino acids located at positions 892, 896 and 899 using site directed mutagenesis according to the manufacturers instructions. The hormone binding domain of pRShAR-wt and pRShAR-TAF1 was then replaced with 1.5Kb Kpn1/BamH1 mutated hormone binding domain DNA fragment. The resulting plasmids were named pRShAR-TAF1 (derived from pRShAR-wt) and pRShAR-null (derived from pRShAR-TAF2). pRShAR-TAF1 contains a mutated form of the AR that carries a series of point mutations that change the glutamic acid residues at positions 892, 896 and serine at position 899 to alanine (AR-TAF1).

The AR-TAF2 plasmid was constructed by excising the 1.5kb AR cDNA fragment generated by cutting with Apa1 and BamH1. The single-stranded DNA overhang generated by Apa1 was removed by treatment with T4 polymerase prior to treatment with BamH1. Another aliquot of pRShAR was treated with Asp718, the ends of the DNA were filled by treatment with Klenow fragment. The DNA was then cut with BamH1. The 4 kb fragment generated was

isolated and ligated with the 1.5 kb Apa1/BamH1 hAR DNA. pRShAR-TAF2 contains a mutated form of the AR that has the N terminal region of the receptor truncated so that amino acids residues 1-506 are missing, effectively removing the TAF1 portion of the receptor.

pRShAR-null contains a mutated form of the AR that carries both the point mutations in the TAF2 region and the truncation of the N terminal region as described above.

Cotransfection assay

CV-1 cells (African green monkey kidney fibroblasts) were cultured in the presence of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% charcoal resin-stripped fetal bovine serum and then transferred to 96-well microtiter plates one day prior to transfection.

Cells were transiently transfected by calcium phosphate coprecipitation (Berger et al. J. Steroid Biochem. Mol. Biol. 41:733, 1992) with pRShAR (1 ng/well), MTV-LUC reporter (100 ng/well), pRS-β-Gal (50 ng/well) and pRS-CAT (filler DNA, 49 ng/well). The receptor plasmid, pRShAR, contains the human AR under constitutive control of the rous sarcoma virus promoter. The reporter plasmid, MTV-LUC, contains the cDNA for LUC under control of the mouse mammary tumor virus (MMTV) long terminal repeat, a conditional promoter containing an androgen response element (Berger, et al. J. Steroid Biochem. Mol. Biol. 41:733, 1992). pRS-β-Gal, coding for constitutive expression of *E. coli* β-galactosidase (β-Gal), was included as an internal control for evaluation of transfection efficiency and compound toxicity. Transfections and subsequent procedures were performed on a Biomek 1000 automated laboratory work station.

Six hours after transfection, media was removed and the cells were washed with phosphate-buffered saline

(PBS). Media containing reference compounds (i.e., DHT and 2-hydroxy flutamide) or test compounds in concentrations ranging from 10^{-12} to 10^{-5} M were added to the cells. Three to four replicates were used for each sample.

After 40 hours, the cells were washed with PBS, lysed with Triton X-100-based buffer and assayed for LUC and β-Gal activities using a luminometer or spectrophotometer, respectively.

Data evaluation was performed using the Oracle relational database management system. For each replicate, the normalized response (NR) was calculated as:

$$\text{LUC response}/\beta\text{-Gal rate}$$

where

β-Gal rate = β-Gal activity $\times 10^{-5}/\beta$-Gal incubation time.

The mean and standard error of the mean (SEM) of the NR were calculated. Data was plotted as the response of the compound compared to the reference compounds over the range of the dose-response curve. For agonist experiments, the effective concentration that produced 50% of the maximum response (EC_{50}) was quantified.

Agonist efficacy was a function (%) of LUC expression relative to the maximum LUC production by the reference agonist, DHT. Antagonist activity was determined by testing the amount of LUC expression in the presence of DHT at its EC_{50} concentration. The concentration of test compound that inhibited 50% of LUC expression induced by DHT was quantified (IC_{50}). In addition, the efficacy of antagonists was determined as a function (%) of maximal inhibition.

Cotransfection studies with the human glucocorticoid receptor (hGR, Giguere, et al. Cell 46:645-652, 1986), human progesterone receptor (hPR-B, Vegeto, et al. Cell 69:703-713, 1992), human mineralocorticoid receptor (hMR) with the MTV-LUC reporter and human estrogen receptor (hER) with the MTV-ERE5-LUC reporter

were carried out as described above for evaluating the cross-reactivity of test compounds.

The receptor constructs were used in transient co-transfection studies (Fig. 18). HepG2 cells were transfected with 10 μg of the reporter plasmid C'9, 5 μg of pRSV-β-gal expression vector as an internal control for transfection efficiency, carrier DNA (pGEM4) added to adjust the total amount of DNA to 20 μg and receptor expression DNA (i.e., pRShAR-wt 200 ng; pRShAR-TAF1 200ng; pRShAR-TAF2 3μg; or pRShAR-null 3μg).

Increasing concentrations of testosterone were added to the HepG2 cells. The procedures and calculations used to determine normalized response follows that described above. The response element used in the reporter plasmid is the C'9 promoter (Adler et al. Mol. Endo. 5:1587-1596, 1991), a fragment of the promoter region of the sex limited protein (SLP) cloned by the polymerase chain reaction from mouse genomic DNA.

Figure 18 demonstrates that in the context of the HEPG2 cell line and C'9 promoter a significant response is obtained with the AR-TAF1 construct relative to wild type AR whereas the AR-TAF2 construct exhibits a very minor response. The AR-null does not exhibit a dose dependent response to hormone. In this example agonist activity can be detected through function of the TAF1 in this context.

Therefore, HepG2 cells, pRShAR-TAF1 and C'9 promoter constitute an assay for an agonist or antagonist which acts through the TAF1 region of AR. With this assay, we can screen agents for partial agonist or antagonist activity mediated by the TAF1 region of hAR.

The development of compounds with androgenic and anti-androgen properties acting through a particular TAF region on certain promoters in certain cellular environments have clinical applications in the treatment of prostate cancer, alopecia, hirsutism, acne, benign prostate hyperplasia, male breast cancer, precocious

puberty and laryngeal carcinoma with little or no side effects. Currently known compounds have side effects. For example, cyprotene acetate (CPA) is a steroidal antiandrogen with progestin activity. Side effects are tests and adrenal suppression, feminization potential teratogen, cardiovascular disorder, liver toxicity, inhibition of libido and bone maturation. In addition, futamide is a nonsteroidal antiandrogen currently used in the treatment of prostate cancer. It is also effective for treating hirsutism. Side effects of futamide include severe gastrointestinal disturbance, hot flashes, gynecomastia, liver toxicity, elevation of serum leutenizing hormone and testosterone levels. Furthermore, casodex is a non-steroidal antiandrogen currently being developed for use in the clinic to treat prostate cancer, hirsutism (in women) and acne. Side effects of casodex include liver toxicity, breast tenderness, gynecomastia, hot flashes, mild back pain.

IV. Glucocorticoid Receptor

Glucocorticoids are potent agents available for the treatment of inflammatory diseases, certain lymphoid cancers and various immunological disorders. However, current therapy is limited by debilitating side effects associated with long term use of glucocorticoids. Side effects include, but are not limited to, hyperglycemia and the resultant diabetes mellitus, osteoporosis, cataracts, fragile skin, weight gain and psychosis. These effects result from the action of glucocorticoid receptor on groups of promoters in specific tissues. In many cases, such as osteoporosis, diabetes and psychosis, the side effects are unrelated to the anti-inflammatory or anti-proliferative effects. Finding GR agonists capable of distinguishing between tissues and/or promoters could limit the effects of the GR agonists to the beneficial anti-inflammatory and anti-proliferative effects.

We have developed assays to detect and distinguish GR agonists that operate through a specific TAF region in certain cellular environments. These assays use cells including a reporter construct containing multimerized glucocorticoid response elements (GRE) upstream from a minimal promoter driving the expression of the reporter gene luciferase. This reporter responds strongly to glucocorticoids in CV1 cells when these cells are transfected with wild type GR.

Receptor expression vectors

Figure 19 is a diagram showing the structural organization of GR-wt, GR-TAF1, GR-TAF2, GR-N-del and GR-null. These expression vectors produce modified forms of GR that selectively inactivate one or more of the transactivation domain of GR.

GR-TAF2 and GR-N-Del plasmids were derived from GR-wt. GR was modified to add a *Not*I restriction site on the 5' side of the DNA binding domain and an *Xho*I site on the 3' side of the DNA binding domain. In GR-TAF2, amino acids 77-262 were excised from GR-wt using *Bgl*II. In GR-N-del, amino acids 9-385 were excised from GR-wt. The procedures were described previously by Hollenberg et al., Cell 49:39-46, 1987, incorporated by reference herein.

To prepare GR-TAF1 plasmid, the TAF2 domain was mutated using a site-directed mutagenesis kit by Clonetech. Amino acid residues 751, 755, 758, and 751+755, 751+758, 755+758 were changed to alanines using the following mutagenesis primers:

751: 5'GCT AAC ATC GCG GGG AAT TC3'
755: 5'GAT GAT TGC AGC AGC TAA CAT C3'
758: 5'CTG ATT GGC GAT GAT TTC AGC3'
751+755: 5'GAT GAT TGC AGC TAA CAT CGC GGG GAA TTC3'
751+758: 5'CTG ATT GGC GAT GAT TTC AGC TAA CAT CGC GGG GAA TTC3'
755+758: 5'CTG ATT GGC GAT GAT TGC AGC TAA C3'

The GR-Null plasmid serves as a negative control that lacks both TAF1 and TAF2 transactivation activity. It is constructed from GR-TAF1 from which the TAF1 domain was excised by *Bgl*II.

To prepare the GR-Gal4 plasmids, the Gal4 DNA binding domain was excised from pBSGalG with KpnI and SalI. The Gal4 DNA binding domain fragment was then inserted into each of the TAF mutants from which the N-terminus and the DNA binding domain of GR have been removed with KpnI and XhoI (see Figure 20).

Reporter plasmids

The reporter plasmids contain either the Murine Mammary Tumor Virus (MMTV-luc) promoter, Complement 3 gene promoter (C3-luc) or TAT3 promoter (i.e., a synthetic promoter consisting of 3 repeats of a Glucocorticoid Response Element (GRE) derived from the Tyrosine Amino Transferase gene fused to the alcohol dehydrogenase TATA box).

Cell culture

CV1, Cos-1, CHO, 293, HepG2, HeLa and HIG82 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). MCF7 were maintained in Iscove's modified Eagle's medium (IMEM) supplemented with 10% FBS.

Transient transfection assay

Transient transfection assays were performed essentially as described for ER except that the cells were incubated with or without hormone for 24 hours.

Dexamethasone binding competition assay

GR proteins were expressed in reticulocyte lysates using a transcription/translation kit from Promega. Aliquots of reticulocyte lysates in binding buffer are incubated with 4-7nM (^{3}H)-dexamethasone and varying

concentrations of unlabelled competing ligand at concentrations ranging from 0 to 10^{-5} M. Incubations were carried out at 4 °C for 16 hours. At the end of the incubation period, GR-bound ligand was absorbed onto hydroxylapatite, pelleted and counted in liquid scintillation cocktail.

The effect of the TAF2 mutations on transactivation was tested by co-transfection (see Figure 21). CV1, Cos-1 (green monkey kidney cells), CHO (Chinese Hamster cells), HeLa (human cervical carcinoma cells), HepG2 (human Hepatocarcinoma cells), MCF7 (human Breast adenocarcinoma cells), HIG82 (rabbit synoviocytes) and 293 (human Embryonal kidney cells) were co-transfected with the Gal4-TAF2 mutants and a reporter plasmid containing Gal4 DNA binding sites fused to luciferase gene. The ability of the TAF2 domain to activate the luciferase gene was detected by measuring chemiluminescence. The data in Figure 21 shows that the mutations in TAF2 inactivated the transactivation activity of TAF2. Similar assays may be conducted to analyze the effects of mutations to other transactivation domains in the GR.

GR-wt, GR-TAF1, GR-TAF2, GR-N-del and GR-Null were cotransfected into CV1 cells with a reporter gene under the regulation of a TAT3 promoter. Dexamethasone was then added to the CV1 cells and the expression level of the reporter gene measured. GR mutants are expressed in CV1 cells. The GR mutations selectively eliminate each transactivation domain but do not significantly change hormone binding. GR-TAF1, GR-N-del and GR-TAF2 have transactivation activity. However, the GR-null receptor has no activation capability, indicating that together the TAF1 and TAF2 mutations eliminate all the transactivation potential from GR.

Figure 22 shows that despite TAF2 transactivation activity was inactivated in GR-TAF1 plasmid, GR-TAF1

still exhibited transactivation activity in CV1 cells on TAT3 promoter. Therefore, CV1 cells, GR-TAF1 plasmid and TAT3 promoter constitute an assay for an agonist or antagonist which acts through the TAF1 region of GR.

Figure 22 also shows that despite TAF1 transactivation activity was inactivated in GR-TAF2 and GR-N-del plasmids by deletion mutations, GR-TAF2 and GR-N-del still exhibited transactivation activity in CV1 cells on TAT3 promoter. Therefore, GR-TAF2 or GR-N-del plasmid, CV1 cells and TAT3 promoter constitute an assay for an agonist or antagonist which acts through the TAF2 region of GR.

The assay we have established for the detection of TAF selective GR agonists or antagonists can utilize any cell line in which either TAF1 or TAF2 is active. The approach described here makes use of the CV1 cell line and mutants of hGR in which either TAF1 or TAF2 is mutated. Each cotransfection assay contains a control β-galactosidase expression vector for transfection efficiency and the TAT3-luciferase reporter.

Individual compounds may be screened in three separate cotransfection assays, the first contains GR-wt, the second contains the GR-TAF1, and the third contains the GR-TAF2 or GR-N-del. Compounds that are active in the wild type assay will be compared in the two mutant assays. All classes of agonists and antagonists may be screened.

Compounds with unique profiles relative to TAF1 or TAF2 identified by the above described assays will generate differences in the tissue or promoter selectivity of the receptor, allowing the determination of which TAF region is associated with the side effects and which is associated with the anti-proliferative or anti-inflammatory activity. The compounds identified by the above described assays may be put to therapeutical uses. For example, if TAF2 is crucial for GR activity in bone cells, then one may selectively eliminate side

effects of GR in osteoclasts and osteoblasts by administering a TAF1 selective agonist screened by the claimed assay to prevent TAF2 activity and reduce the osteoporosis associated with glucocorticoid therapy.

V. Pharmaceutical Compositions

The present invention also encompasses pharmaceutical compositions prepared for storage and subsequent administration, which have a pharmaceutically effective amount of the products disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired,

absorption enhancing preparations (e.g., liposomes) may be utilized.

The pharmaceutically effective amount of the composition required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

In practicing the methods of the invention, the products or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized in vivo, ordinarily in a mammal, preferably in a human, or in vitro. In employing them in vivo, the products or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms.

As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. In non-human animal studies, applications of products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear.

The dosage for the products of the present invention can range broadly depending upon the desired affects and the therapeutic indication. Typically, dosages may be between about 10 μg/kg and 100 mg/kg body weight, preferably between about 100 μg/kg and 10 mg/kg body weight. Administration is preferably oral on a daily basis.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous,

intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and

processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

All publications referenced are incorporated by reference herein, including the nucleic acid sequences and amino acid sequences listed in each publication.

All the compounds disclosed and referred to in the publications mentioned above are incorporated by reference herein, including those compounds disclosed and referred to in articles cited by the publications mentioned above.

Other embodiments of this invention are disclosed in the following claims.

Ex Vivo Formation of Osteoclasts

TRAP(+)MNC
(% Control)

	Sham	OVX	OVX +17βE2	OVX +Keoxifen
Expt. 1	100	147.8[a]	84.3[c]	99.3[c]
Expt. 2	100	154.0[b]	119.4[c]	78.2[a]

[a]Significantly different from sham, $p<0.02$.
[b]Significantly different from sham, $p<0.001$.
[c]No significant difference from sham.

Table 1

Bone marrow from sham (control), OVX (ovariectomized rats), OVX plus estrogen, and OVX plus keoxifene treated rats were evaluated for osteoclastic potential in the coculture assay. Bone marrow was combined with primary osteoblasts in the presence of 1,25-dihydroxyvitamin D3 and parathyroid hormone for 8 days and scored for the number of tartrate acid phosphatase resistant multinucleated cells (TRAP + MNC). The number of TRAP + MNC in the sham operated animals was set at 100%.

TABLE 2

List of Candidate Compounds

	CANDIDATE COMPOUNDS	REFERENCES
1)	Compounds Modulating Glucocorticoids	*p.1213 ff, and references therein WO/92/16546, PCT/US92/02024 WO92/16658, PCT/US92/02014 US 4,981,787 US 5,071,773 R. Evans, *Science* 240:889-895 (1988)
2)	Estrogens-agonists & antagonists	*p. 1193 ff and references therein
3)	Androgens-agonists & antagonists	*p. 1208 ff, and references therein US 4,144,270 US 3,847,988 US 3,995,060
4)	Progestins-agonists & antagonists Non-steroid progestins	*p. 1200 ff, and references therein PCT/US93/03909 PCT/US93/10086 WO 94/24080
5)	Mineralocorticoids-agonists & antagonists	*p. 1213 ff, and references therein
6)	Nonsteroidal anti-inflammatory drugs	**

*Intracellular receptor general reference Comprehensive Medicinal Chemistry "The Rational Design, Mechanistic Study and Therapeutic Applications of Chemical Compounds," C. Hamsch, P.G. Sammes, John B. Taylor and John C. Emmett Vol. 3-Membrane and Receptors, Pregammon Press, Oxford, Ch. 16.3 Steroid Hormone Receptors pp. 1176-1226.

**Negrel et al., *Biochem. J* 257:399-405 (1989)

Claims:

1. Method for identifying a receptor agonist or antagonist, comprising the steps of:

(A) providing a cell comprising a first nucleic acid encoding an intracellular receptor and a second nucleic acid encoding a reporter gene operatively linked to a promoter, wherein said intracellular receptor comprises a first TAF region and a second TAF region, said first TAF region is able to activate transcription from said promoter, said second TAF region is not able to activate transcription from said promoter but retains the functional context of said second TAF region, and said reporter gene is transcribed when said promoter is activated by said first TAF region;

furthermore, said cell is unable to exhibit transcription from said promoter in the presence of a receptor containing said second TAF region alone but able to exhibit transcription from said promoter in the presence of a receptor containing said first TAF region;

(B) contacting said cell with an agent under conditions in which contact of said cell with a known agonist or antagonist of said intracellular receptor increases or decreases the transcription of said reporter gene from said promoter, respectively; and

(C) measuring the level of increase or decrease of the expression product of said reporter gene as an indication of the agonist or antagonist activity of said agent.

2. The method of claim 1, wherein said second TAF region is mutated.

3. The method of claim 1, wherein said receptor is selected from the group consisting of ER, GR, AR, PR, MR, RAR, RXR and PPAR.

4. The method of claim 2, wherein said first TAF region is a TAF1 region selected from ER, GR, AR or PR, and said second TAF region is a TAF2 region selected from ER, GR, AR or PR.

5. The method of claim 2, wherein said first TAF region is a TAF2 region selected from ER, GR, AR or PR, and said second TAF region is a TAF1 region selected from ER, GR, AR or PR.

6. The method of claim 1, wherein said agent is a human hormone agonist or antagonist.

7. The method of claim 1, wherein said agent is a compound other than keoxifene with a keoxifene-like transcriptional profile.

8. The method of claim 1, wherein said cell is selected from the group consisting of HepG2, MCF-10, CV1 and HeLa.

9. The method of claim 1, wherein said cell is a liver cell.

10. The method of claim 1, wherein said promoter is selected from the group consisting of C3, MMTV, TAT and C'9.

11. The method of claim 1, wherein said promoter is a C3 promoter.

12. Method for identifying a receptor agonist, comprising the steps of:

(A) providing a cell comprising

(i) a first nucleic acid encoding an intracellular receptor having a functional first TAF region and a functional second TAF region; and

(ii) a second nucleic acid encoding a reporter gene operatively linked to a promoter, which is activated in the presence of an agonist that acts through one of said TAF regions but not both;

furthermore, said cell is able to exhibit transcription from said promoter in the presence of a receptor containing both said TAF regions, but unable to exhibit transcription from said promoter in the presence of a receptor having said first TAF or said second TAF region alone;

(B) contacting said cell with a potential agonist under conditions in which contact of said cell with a known agonist of said receptor increases transcription from said promoter and the level of the product of said reporter construct; and

(C) measuring the level of increase of said product of said reporter construct as an indication of the agonist activity of said potential agonist.

13. The method of claim 12, wherein said cell is a liver cell, and said promotor is a C3 promotor.

14. The method of claim 12, wherein said agent is a compound other than keoxifene with a keoxifene-like transcriptional profile.

15. A method for treating a patient with an estrogen related disease comprising administering to said patient a chemical compound other than keoxifene having a keoxifene like transcriptional profile.

16. The method of claim 15, wherein said disease is osteoporosis.

17. The method of claim 15, wherein said disease is uterine cancer.

18. The method of claim 15, wherein said disease is breast cancer.

19. The method of claim 15, wherein said treating comprises administering a pharmaceutically acceptable amount of said compound to said patient.

20. The method of claim 19, wherein said administering is performed orally.

21. The method of claim 15, wherein said compound has greater potency than keoxifene.

FIG. 1a.

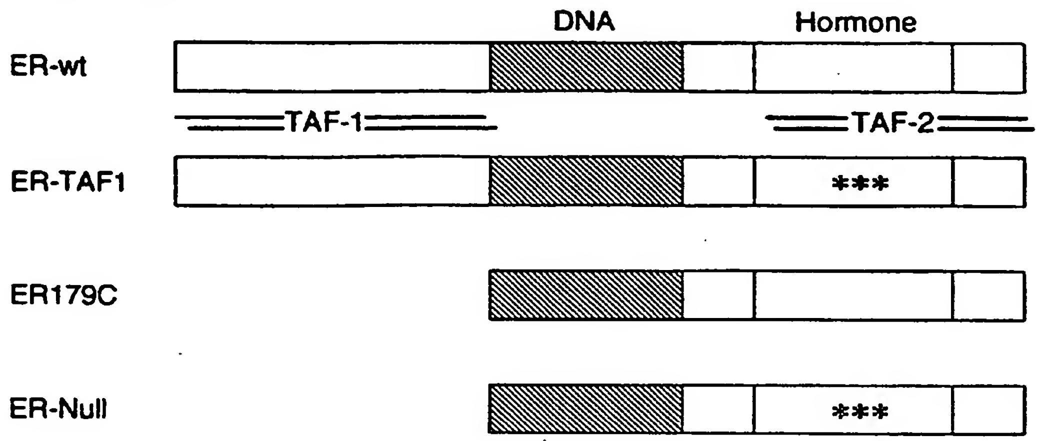

DNA
Hormone
ER-wt
TAF-1
TAF-2
ER-TAF1
***
ER179C
ER-Null
***

FIG. 1b.

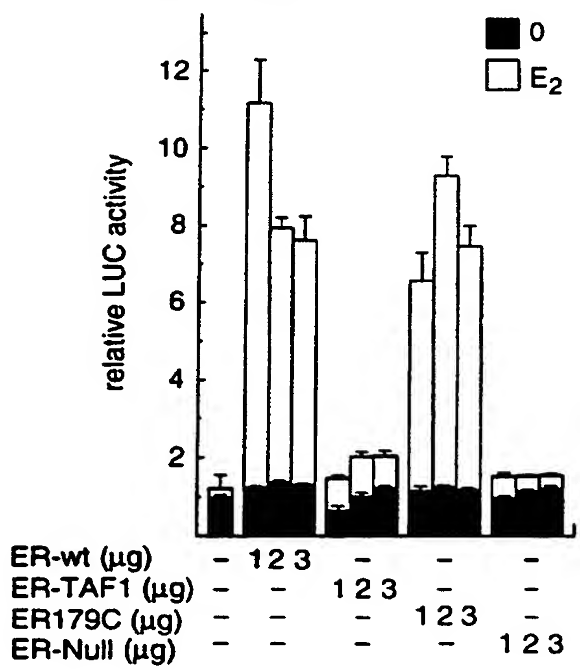

0
E_2
relative LUC activity
12
10
8
6
4
2
ER-wt (µg) - 1 2 3 - - -
ER-TAF1 (µg) - - 1 2 3 - -
ER179C (µg) - - - 1 2 3 -
ER-Null (µg) - - - - 1 2 3

FIG. 1c.

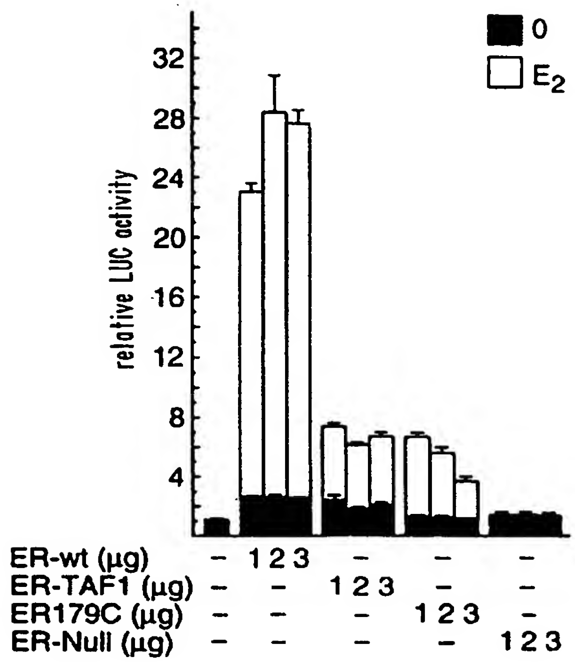

0
E_2
relative LUC activity
32
28
24
20
16
12
8
4
ER-wt (µg) – 1 2 3 – – –
ER-TAF1 (µg) – – 1 2 3 – –
ER179C (µg) – – – 1 2 3 –
ER-Null (µg) – – – – 1 2 3

FIG. 1d.

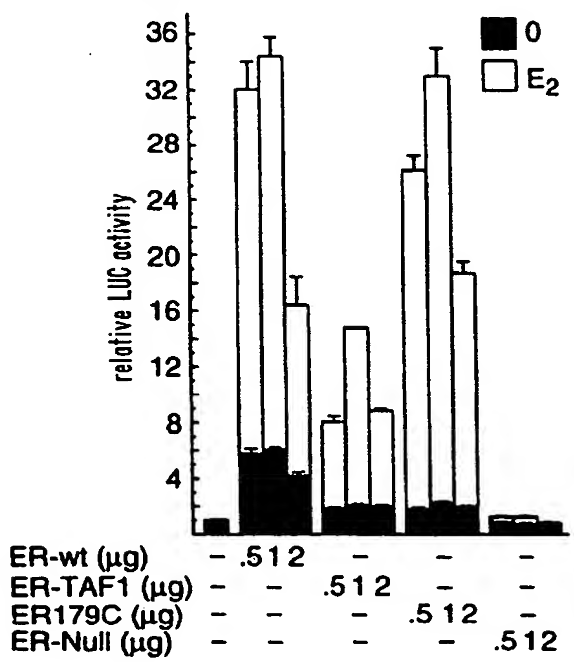

0
E_2
relative LUC activity
36
32
28
24
20
16
12
8
4
ER-wt (µg) – .5 1 2 – – –
ER-TAF1 (µg) – – .5 1 2 – –
ER179C (µg) – – – .5 1 2 –
ER-Null (µg) – – – – .5 1 2

FIG. 2a.

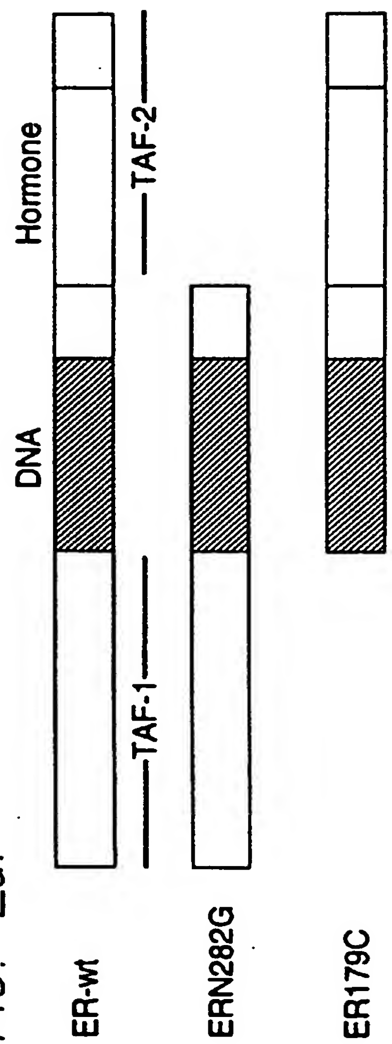


FIG. 2b.

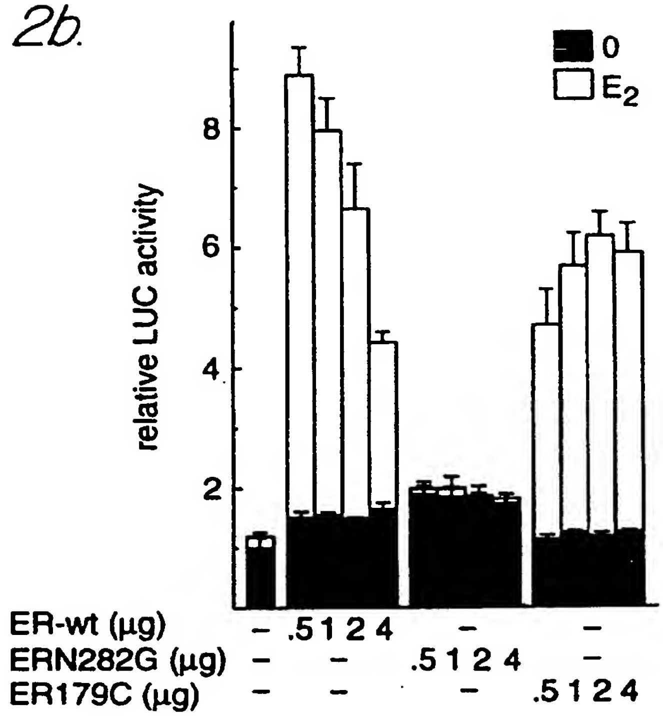

0
E_2
relative LUC activity
2
4
6
8
ER-wt (µg) – .5 1 2 4 – –
ERN282G (µg) – – .5 1 2 4 –
ER179C (µg) – – – .5 1 2 4

FIG. 2c.

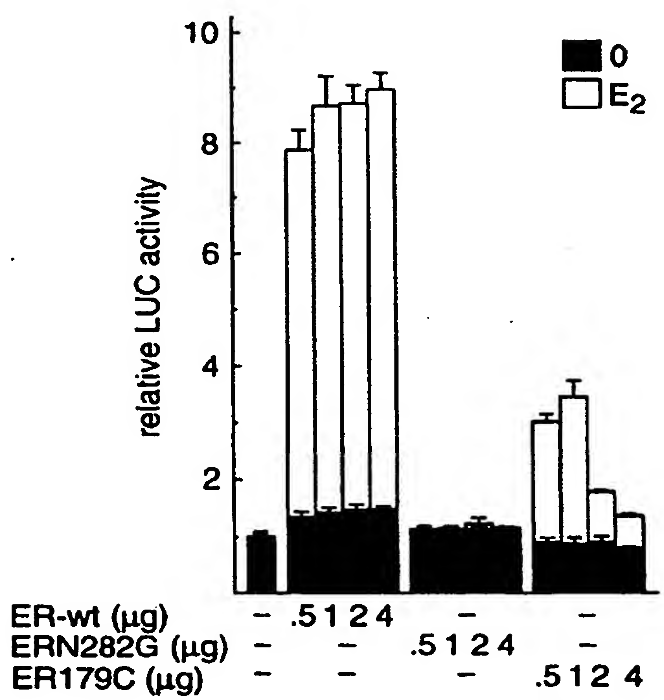
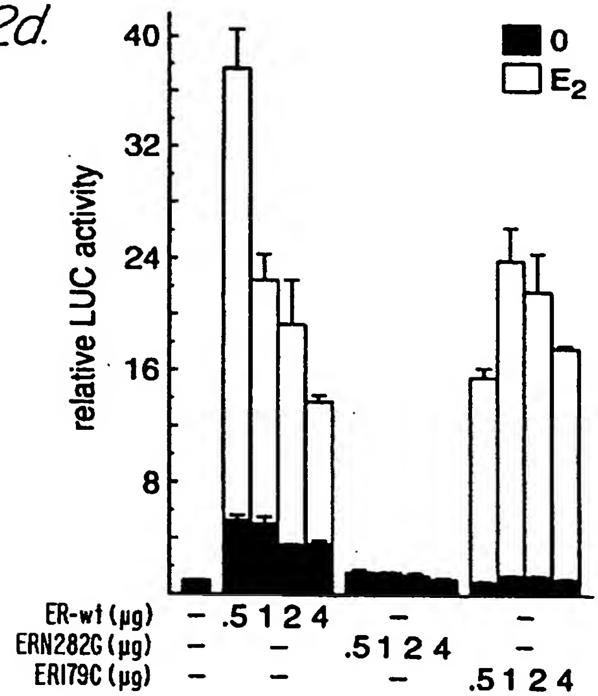

0
E_2
relative LUC activity
2
4
6
8
10
ER-wt (µg) – .5 1 2 4 – –
ERN282G (µg) – – .5 1 2 4 –
ER179C (µg) – – – .5 1 2 4

FIG. 2d.



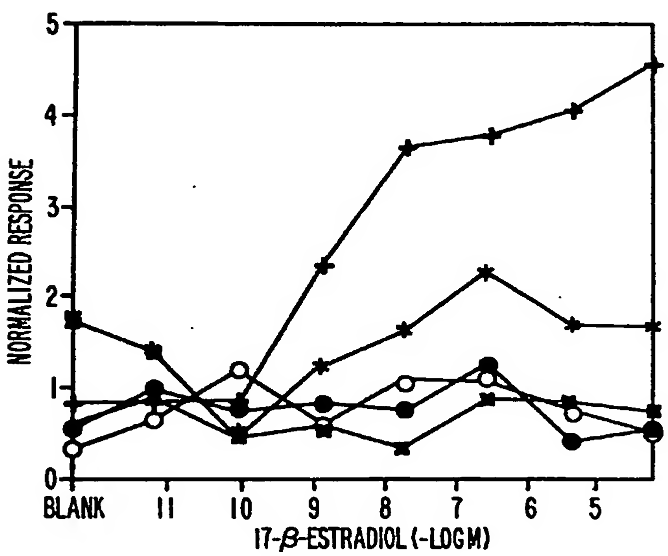


FIG. 3a.

ER-wt +
ER-TAF1 *
ERI79C O
ER-null X

FIG. 3b.

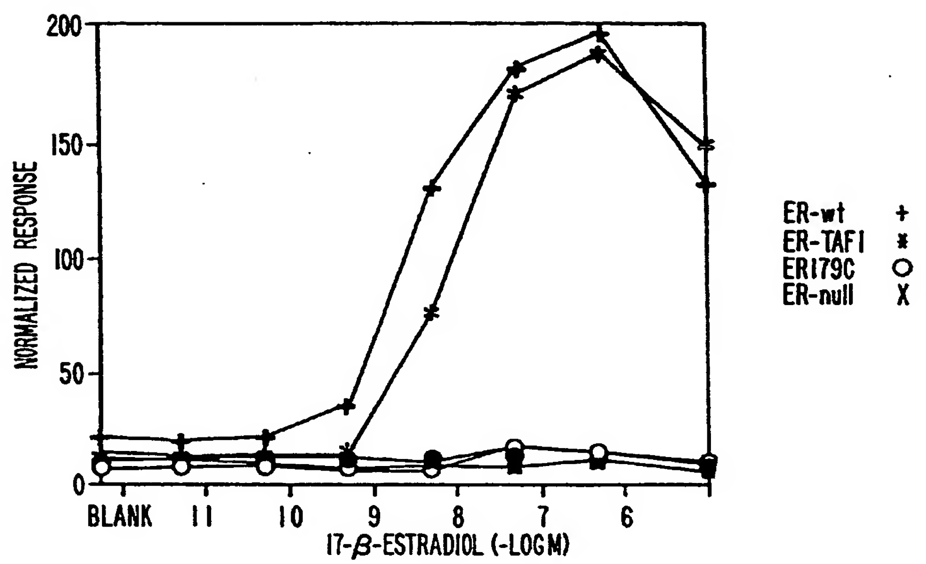

200
150
100
50
0
NORMALIZED RESPONSE
BLANK
11
10
9
8
7
6
17-β-ESTRADIOL (-LOGM)
ER-wt +
ER-TAF1 *
ER179C O
ER-null X

FIG. 3c.

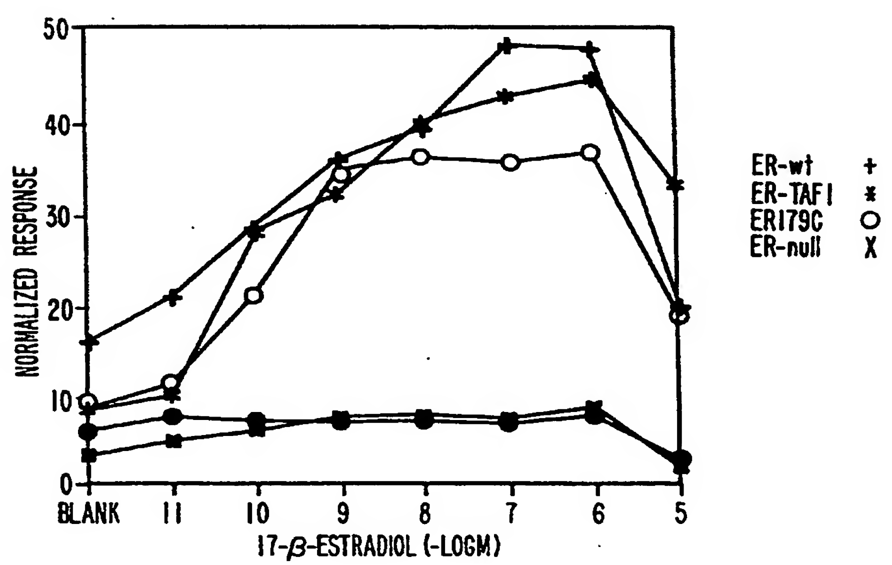

50
40
30
20
10
0
NORMALIZED RESPONSE
BLANK
11
10
9
8
7
6
5
17-β-ESTRADIOL (-LOGM)
ER-wt +
ER-TAF1 *
ER179C O
ER-null X

FIG. 4a.

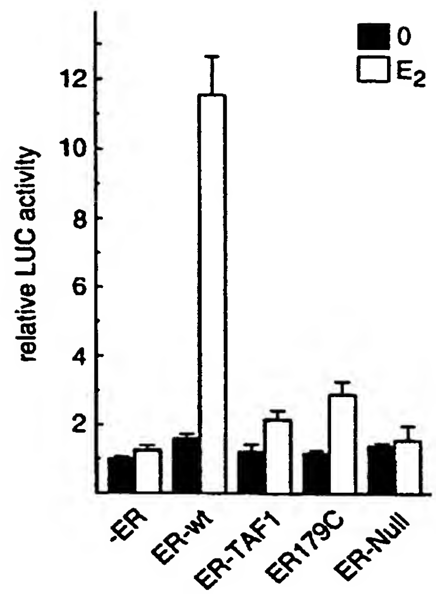


FIG. 4b.

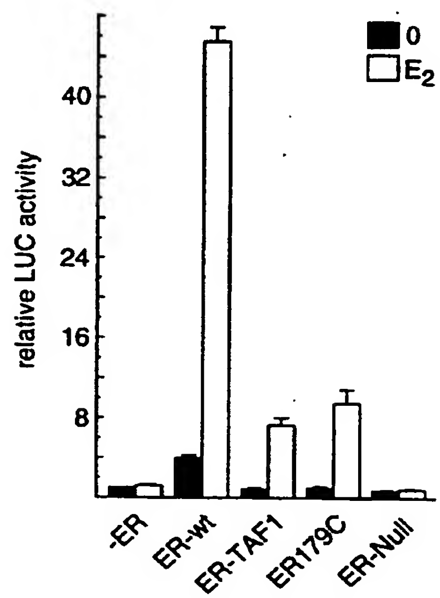


FIG. 4c.

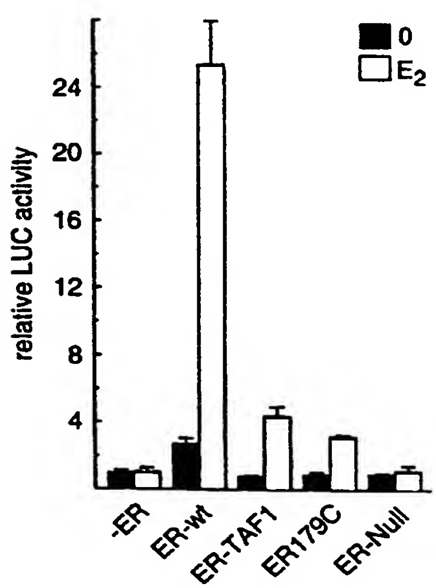
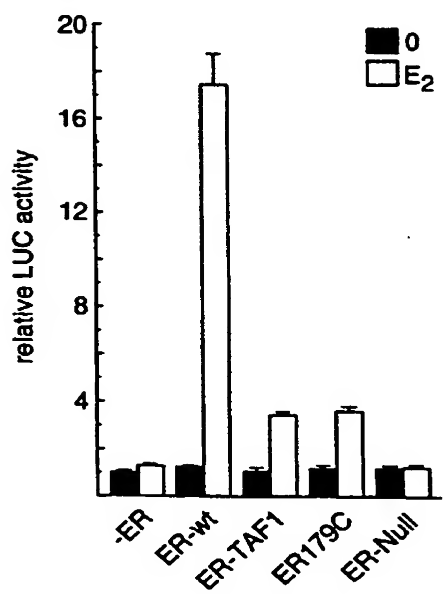


FIG. 4d.



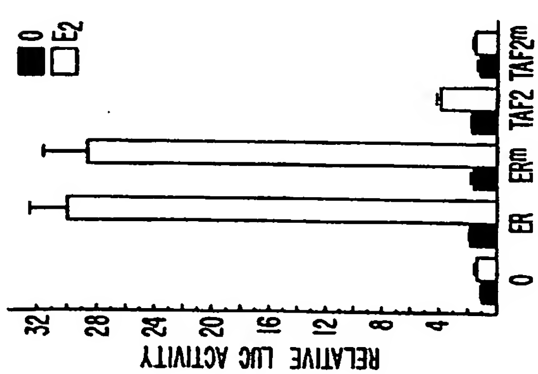


FIG. 5a.

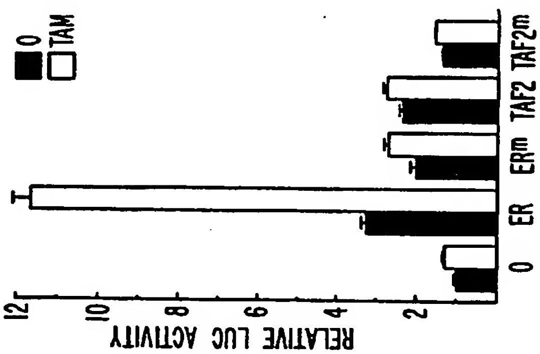


FIG. 5b.

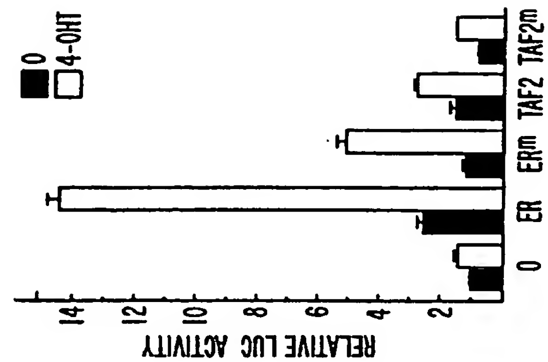


FIG. 5c.

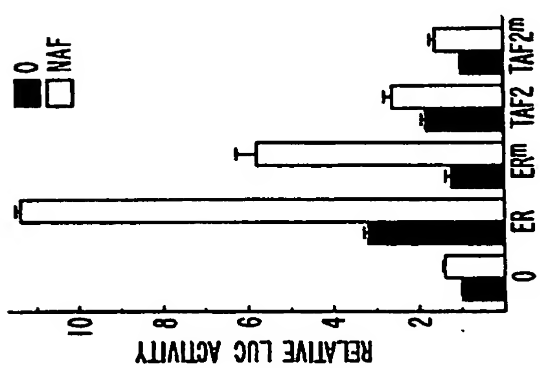


FIG. 5d.

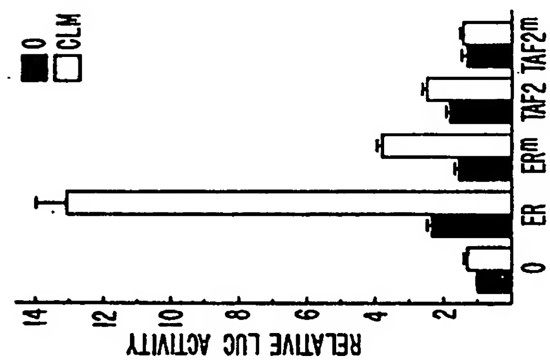


FIG. 5e.

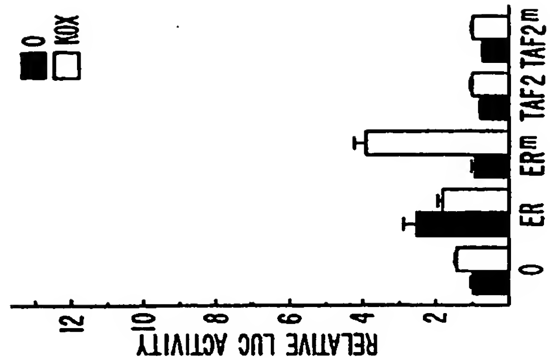


FIG. 5f.

FIG. 6b.

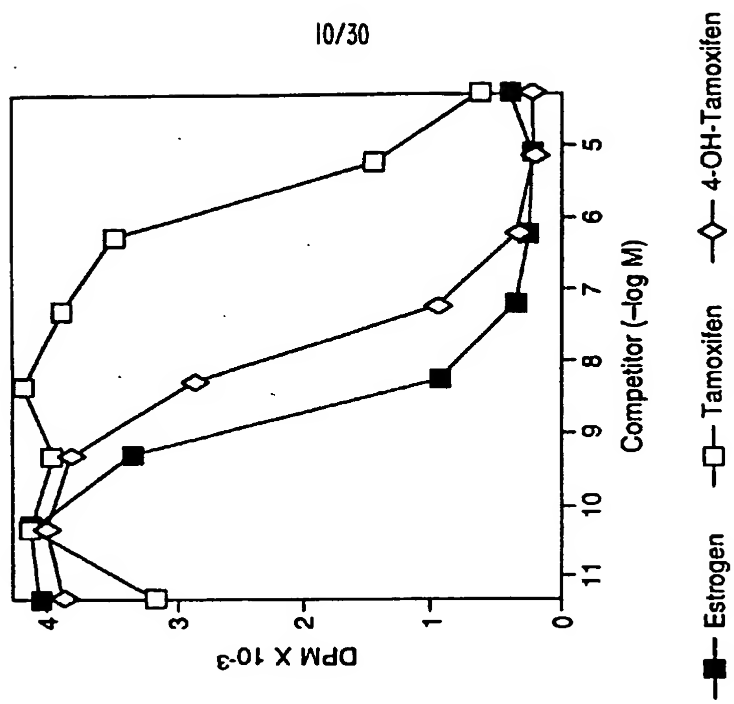

DPM X 10-3
4
3
2
1
0
11
10
9
8
7
6
5
Competitor (−log M)
Estrogen
Tamoxifen
4-OH-Tamoxifen

FIG. 6a.

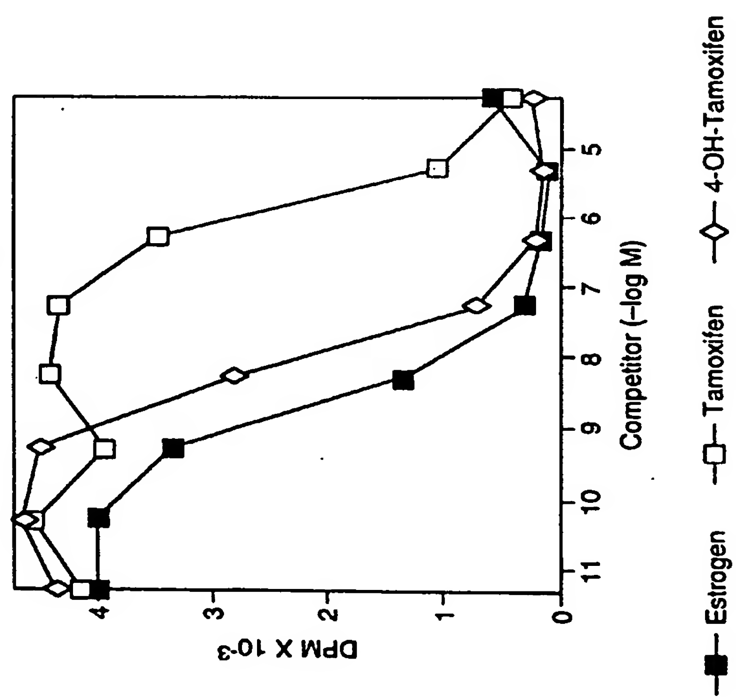

DPM X 10-3
4
3
2
1
0
11
10
9
8
7
6
5
Competitor (−log M)
Estrogen
Tamoxifen
4-OH-Tamoxifen

FIG. 7c.

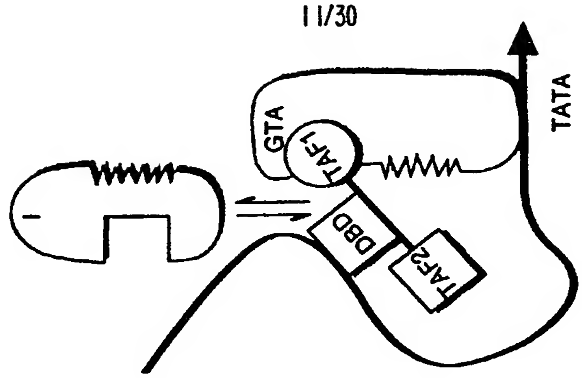


FIG. 7b.

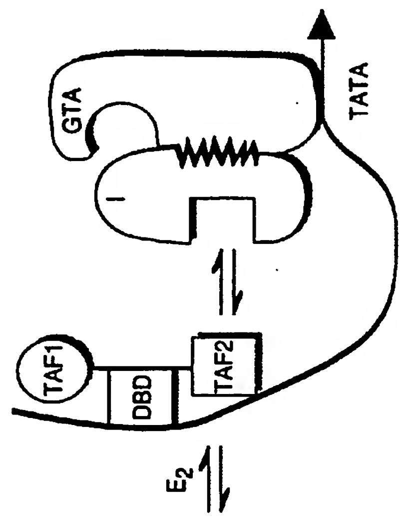


FIG. 7a.

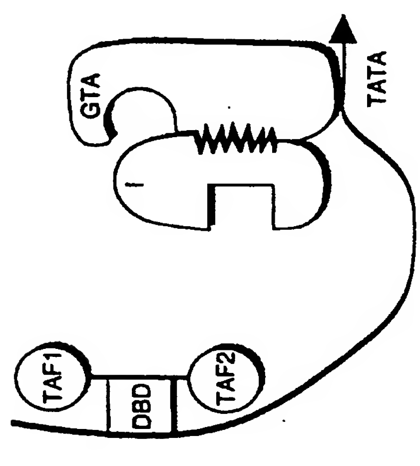


FIG. 8a.

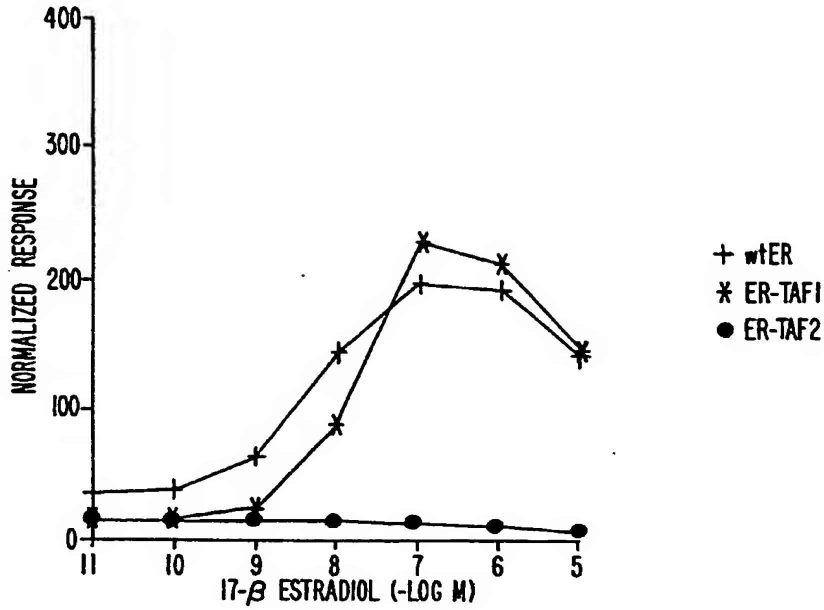

400
300
200
100
0
NORMALIZED RESPONSE
11 10 9 8 7 6 5
17-β ESTRADIOL (-LOG M)
wtER
ER-TAF1
ER-TAF2

FIG. 8b.

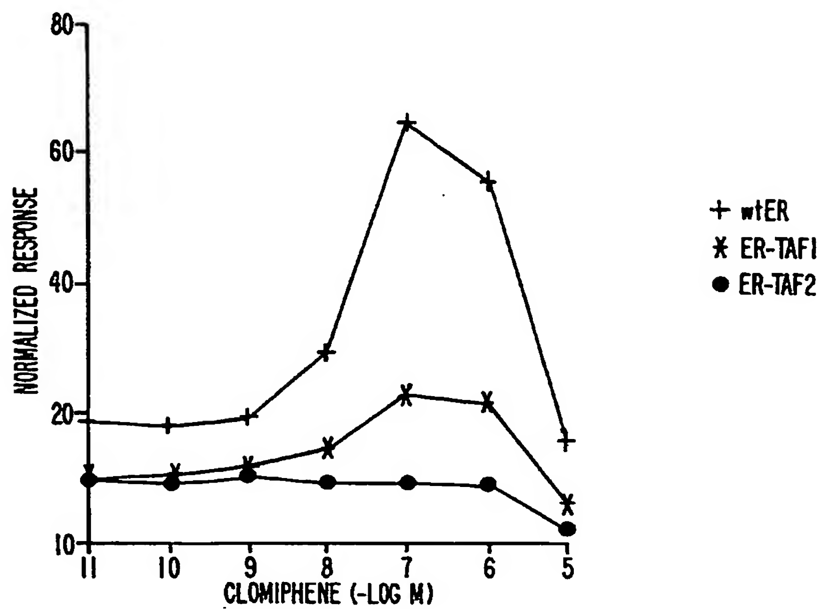

80
60
40
20
10
NORMALIZED RESPONSE
11 10 9 8 7 6 5
CLOMIPHENE (-LOG M)
wtER
ER-TAF1
ER-TAF2

FIG. 8c.

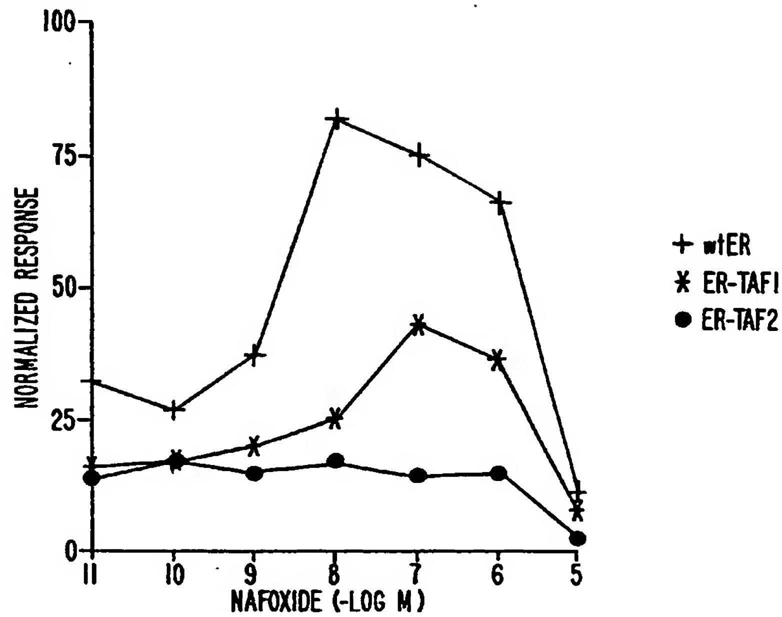

100
75
50
25
0
NORMALIZED RESPONSE
11
10
9
8
7
6
5
NAFOXIDE (-LOG M)
+ wtER
* ER-TAF1
● ER-TAF2

FIG. 8d.

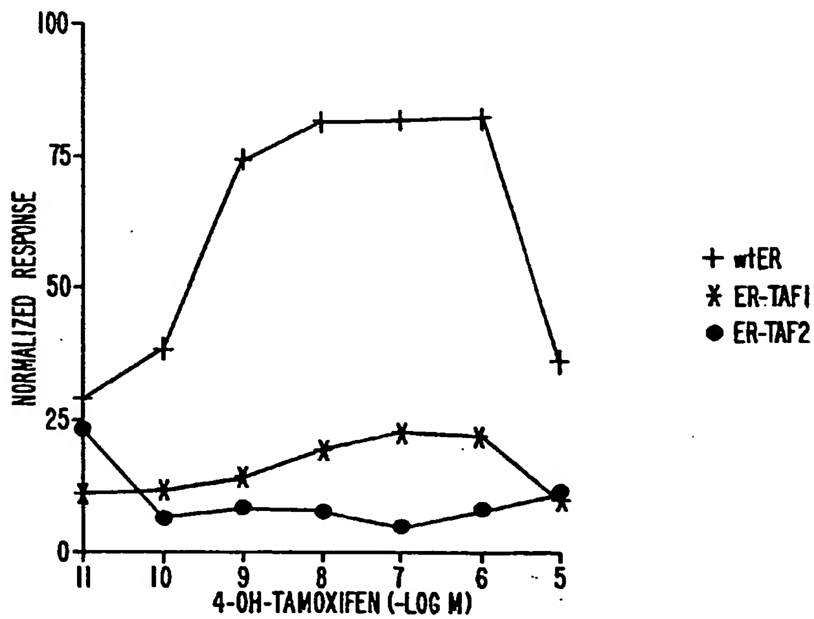

100
75
50
25
0
NORMALIZED RESPONSE
11
10
9
8
7
6
5
4-OH-TAMOXIFEN (-LOG M)
+ wtER
* ER-TAF1
● ER-TAF2

FIG. 8e.

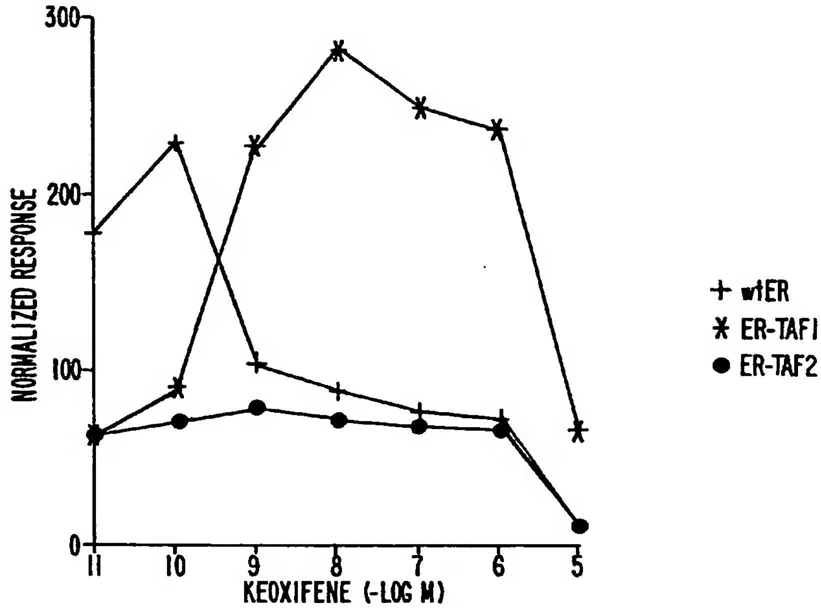

300
200
100
0
NORMALIZED RESPONSE
11
10
9
8
7
6
5
KEOXIFENE (-LOG M)
+ wtER
* ER-TAF1
● ER-TAF2

FIG. 10.

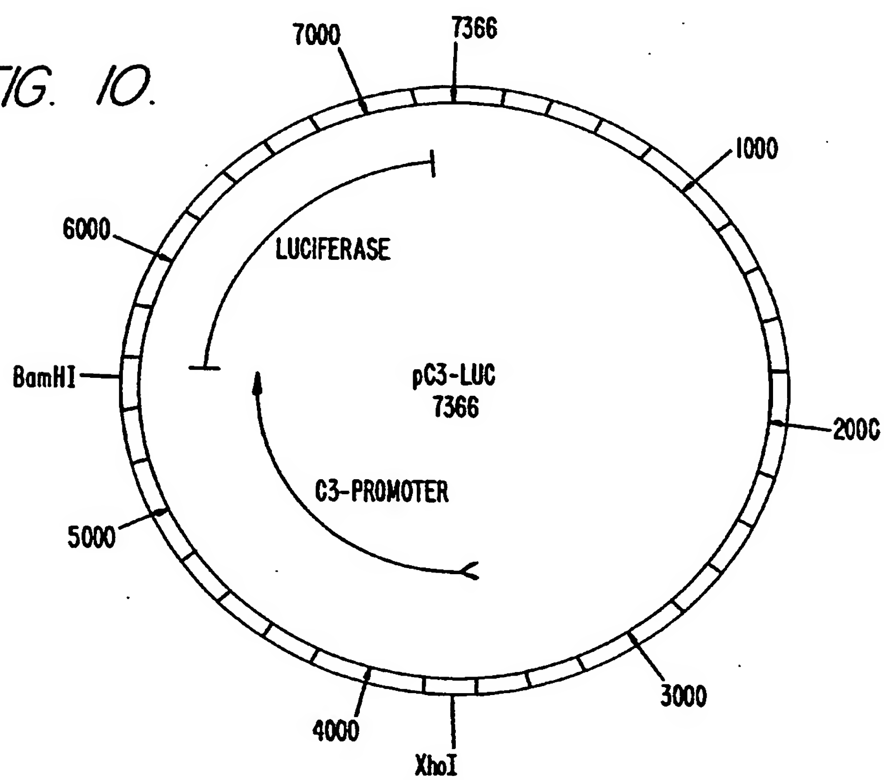

7000
7366
1000
6000
LUCIFERASE
BamHI
pC3-LUC
7366
2000
C3-PROMOTER
5000
3000
4000
XhoI

FIG. 9.

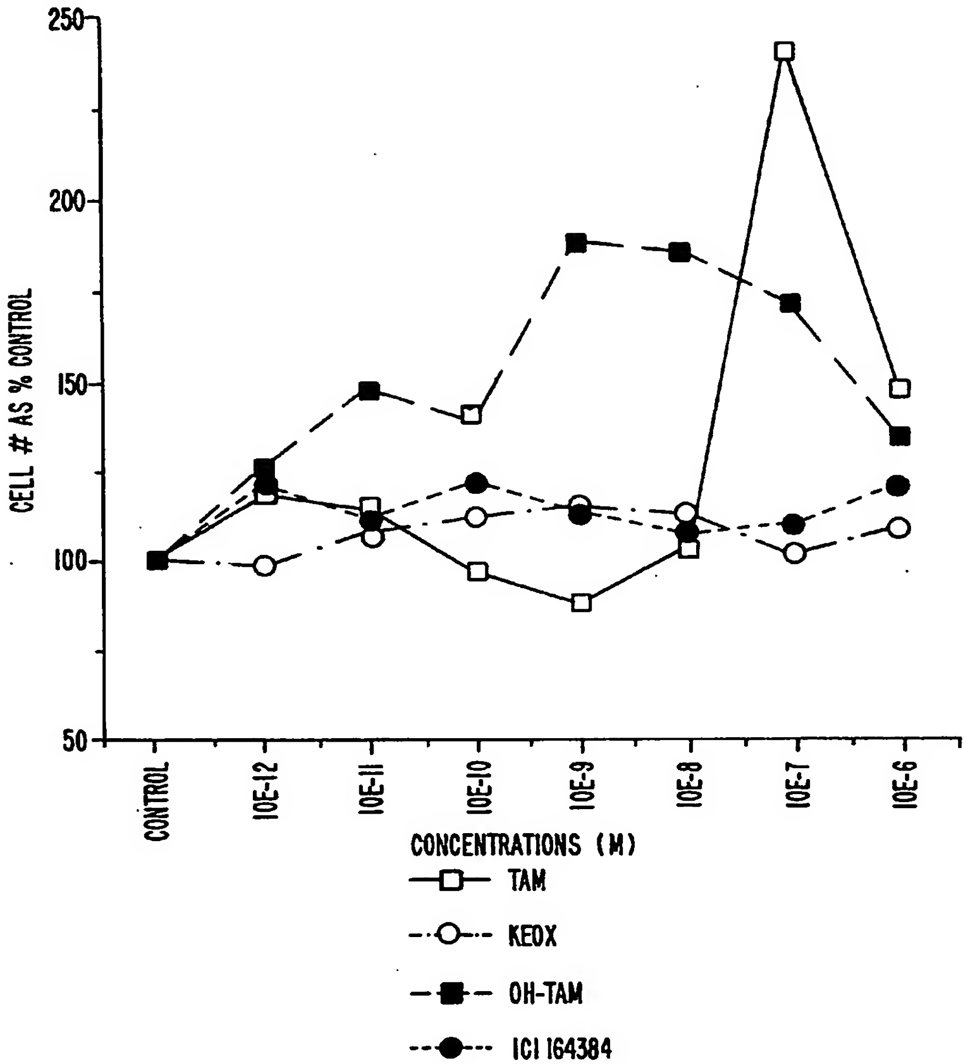

250
200
150
100
50
CELL # AS % CONTROL
CONTROL
10E-12
10E-11
10E-10
10E-9
10E-8
10E-7
10E-6
CONCENTRATIONS (M)
TAM
KEOX
OH-TAM
ICI 164384

FIG. 11.

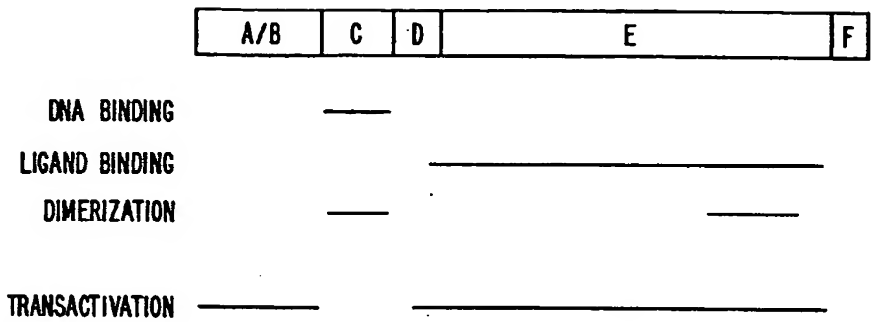

A/B
C
D
E
F
DNA BINDING
LIGAND BINDING
DIMERIZATION
TRANSACTIVATION

FIG. 12.

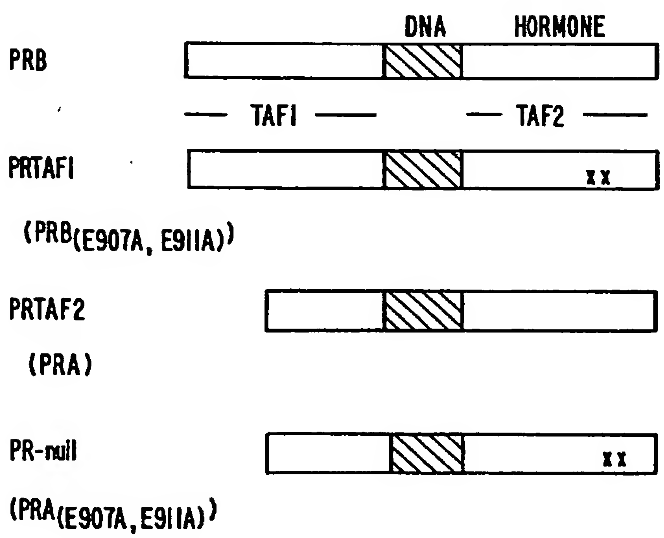

DNA
HORMONE
PRB
TAF1
TAF2
PRTAF1
xx
(PRB(E907A, E911A))
PRTAF2
(PRA)
PR-null
xx
(PRA(E907A, E911A))

FIG. 13a.

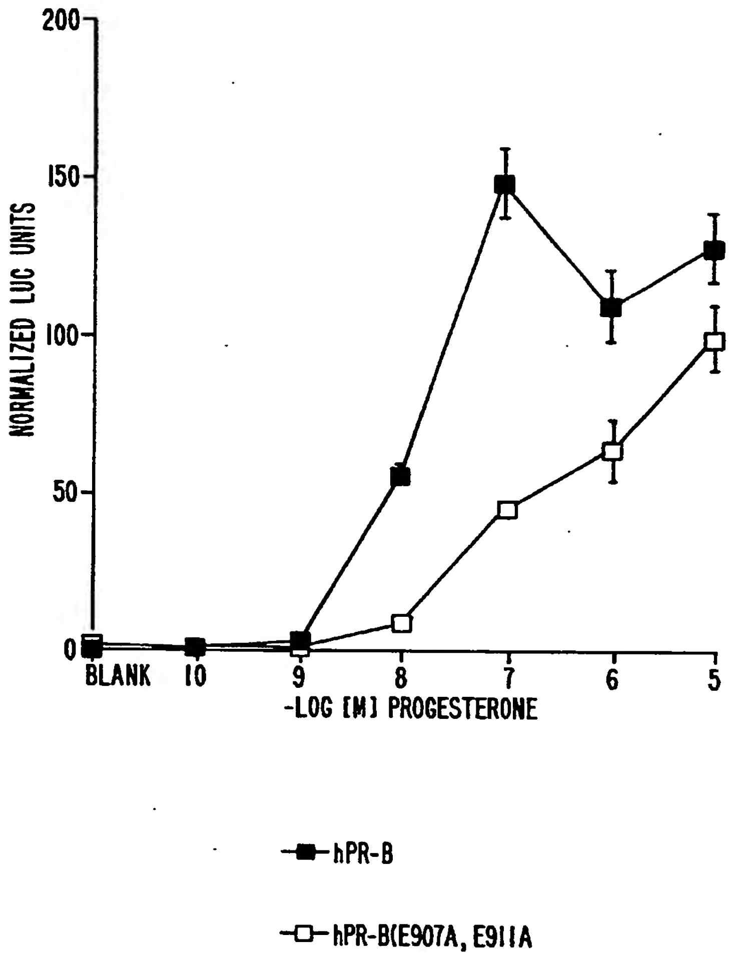
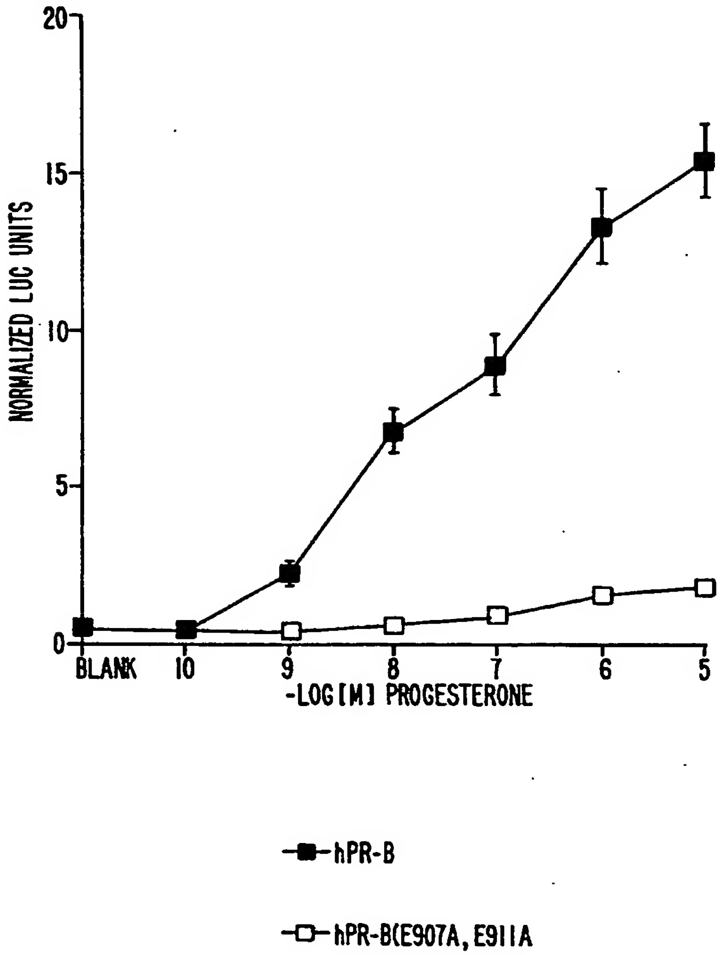

200
150
100
50
0
NORMALIZED LUC UNITS
BLANK
10
9
8
7
6
5
-LOG [M] PROGESTERONE
hPR-B
hPR-B(E907A, E911A

FIG. 13b.


20
15
10
5
0
NORMALIZED LUC UNITS
BLANK
10
9
8
7
6
5
-LOG[M] PROGESTERONE
hPR-B
hPR-B(E907A, E911A

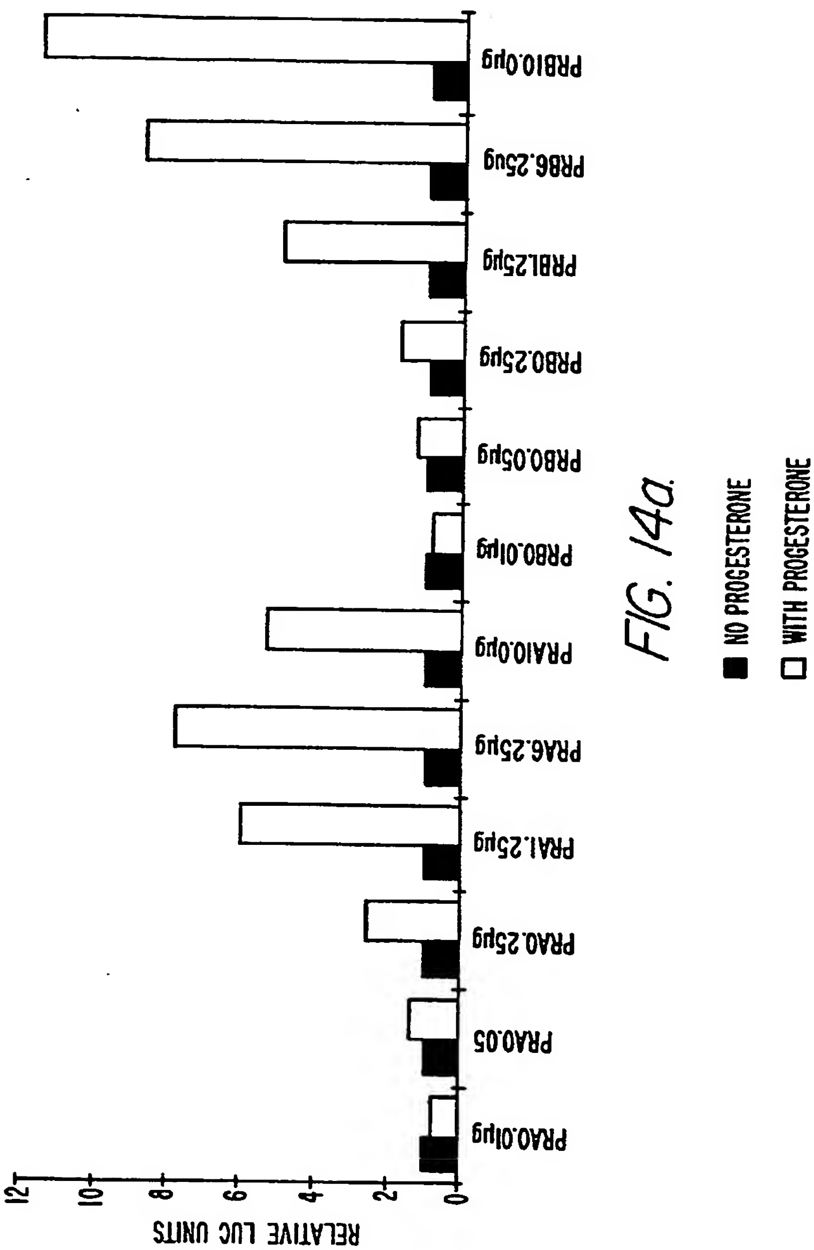

RELATIVE LUC UNITS
0
2
4
6
8
10
12
PRA0.01µg
PRA0.05
PRA0.25µg
PRA1.25µg
PRA6.25µg
PRA10.0µg
PRB0.01µg
PRB0.05µg
PRB0.25µg
PRB1.25µg
PRB6.25µg
PRB10.0µg
NO PROGESTERONE
WITH PROGESTERONE

FIG. 14a.

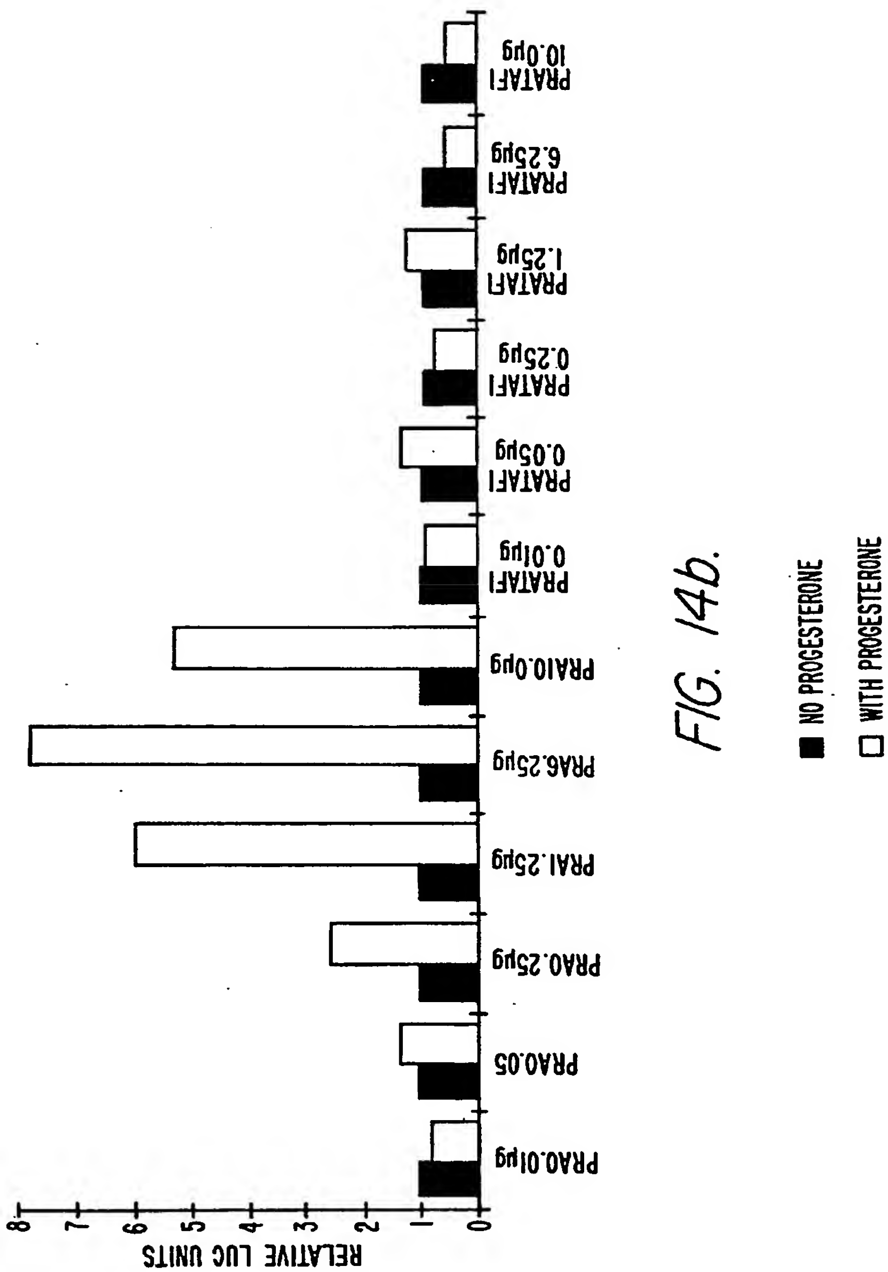

RELATIVE LUC UNITS
0
1
2
3
4
5
6
7
8
PRA0.01µg
PRA0.05
PRA0.25µg
PRA1.25µg
PRA6.25µg
PRA10.0µg
PRATAFI 0.01µg
PRATAFI 0.05µg
PRATAFI 0.25µg
PRATAFI 1.25µg
PRATAFI 6.25µg
PRATAFI 10.0µg
NO PROGESTERONE
WITH PROGESTERONE

FIG. 14b.

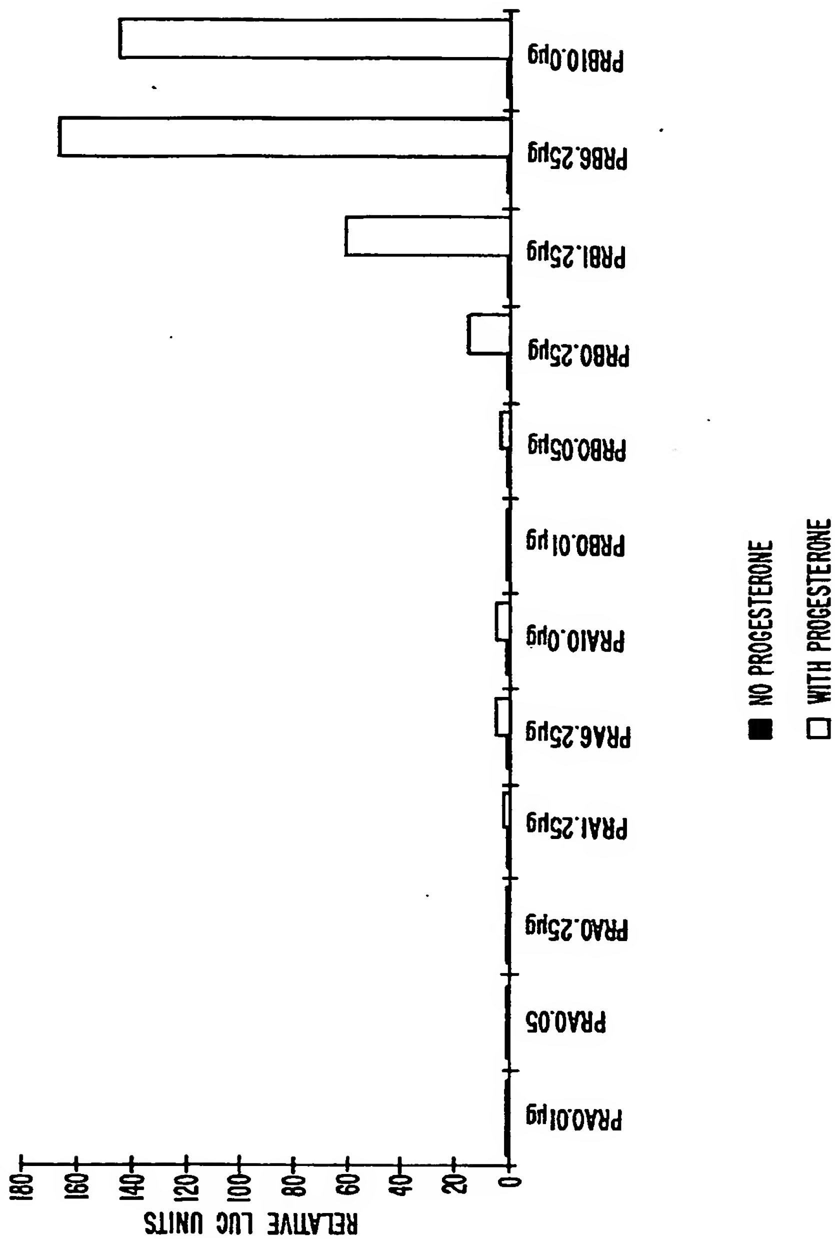

RELATIVE LUC UNITS
0
20
40
60
80
100
120
140
160
180
PRA0.01µg
PRA0.05
PRA0.25µg
PRA1.25µg
PRA6.25µg
PRA10.0µg
PRB0.01µg
PRB0.05µg
PRB0.25µg
PRB1.25µg
PRB6.25µg
PRB10.0µg
NO PROGESTERONE
WITH PROGESTERONE

FIG. 15a.

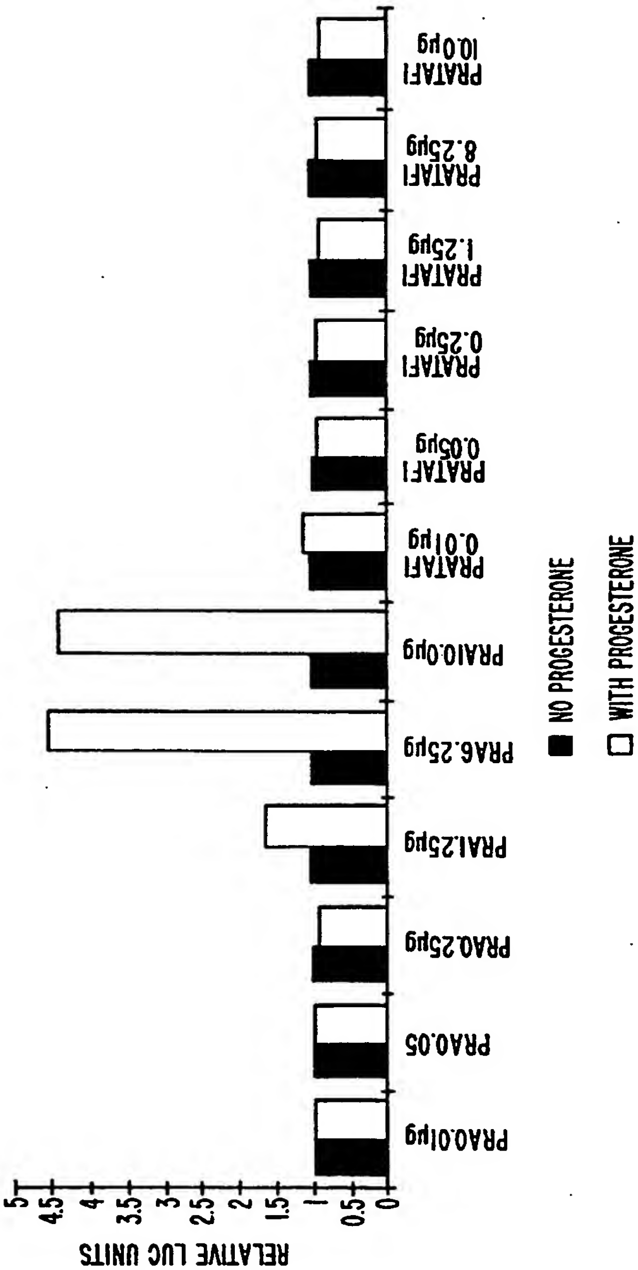

RELATIVE LUC UNITS
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
PRA0.01µg
PRA0.05
PRA0.25µg
PRA1.25µg
PRA6.25µg
PRA10.0µg
PRATAFI 0.01µg
PRATAFI 0.05µg
PRATAFI 0.25µg
PRATAFI 1.25µg
PRATAFI 8.25µg
PRATAFI 10.0µg
NO PROGESTERONE
WITH PROGESTERONE

FIG. 15b.

FIG. 16a.

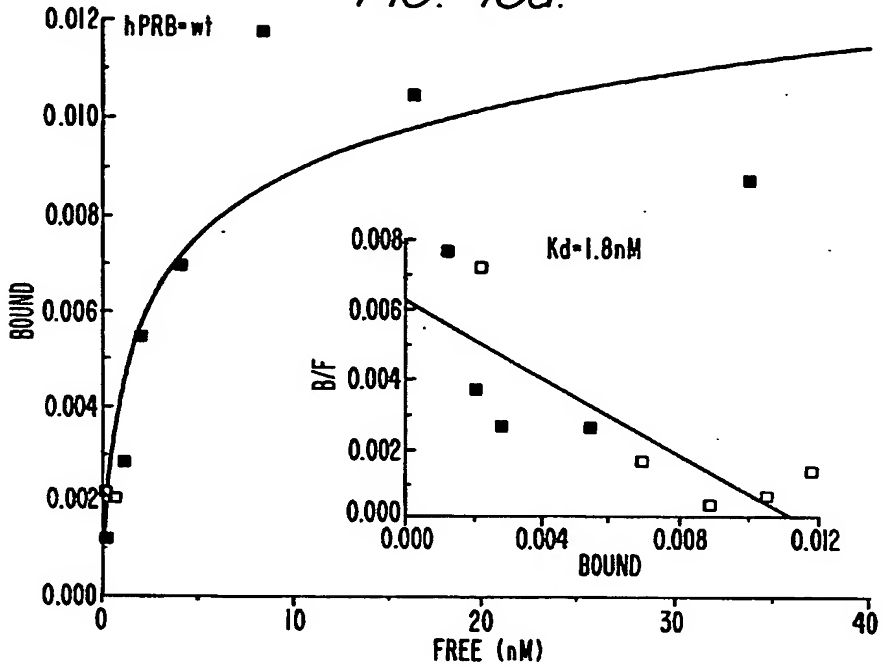


FIG. 16b.

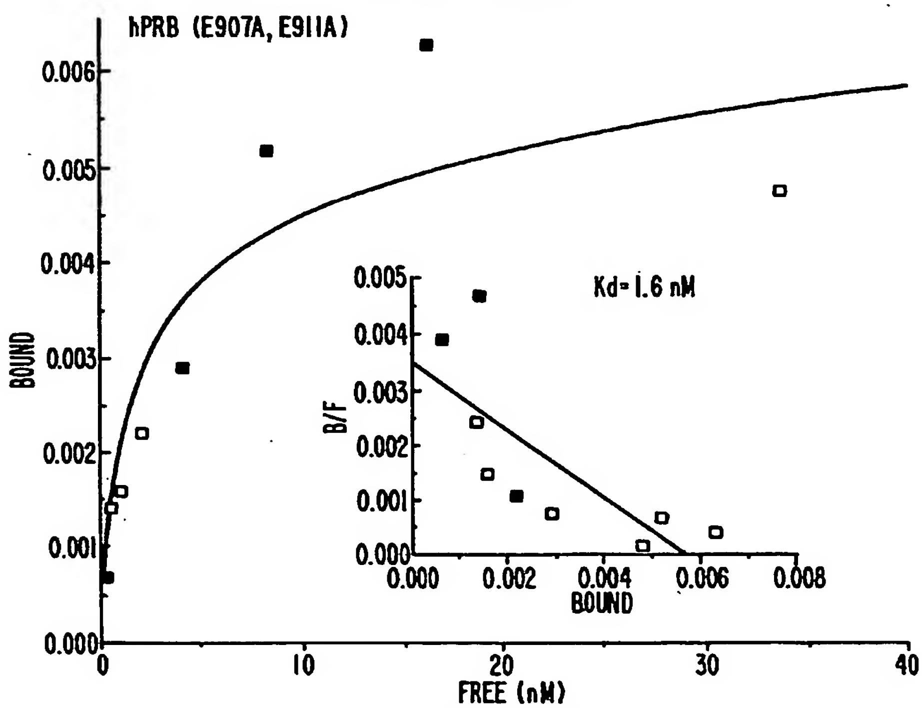


FIG. 16c.

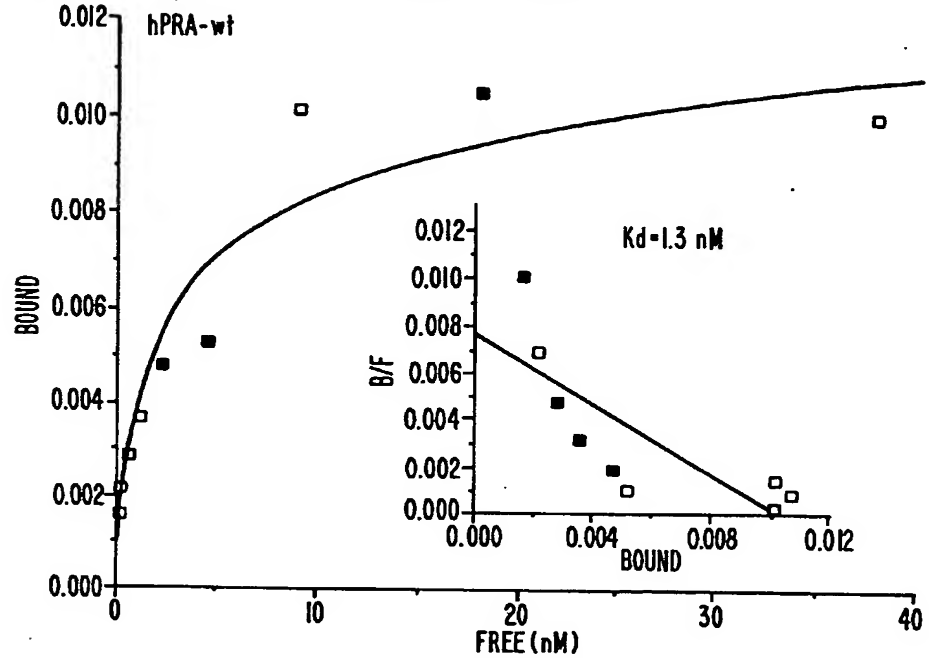


FIG. 16d.

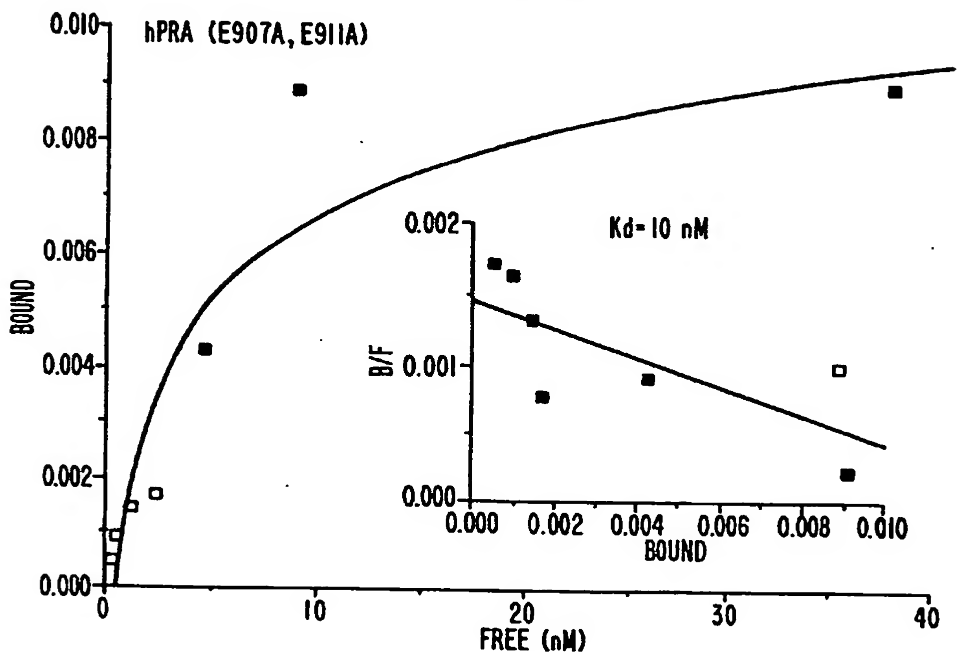


FIG. 17.

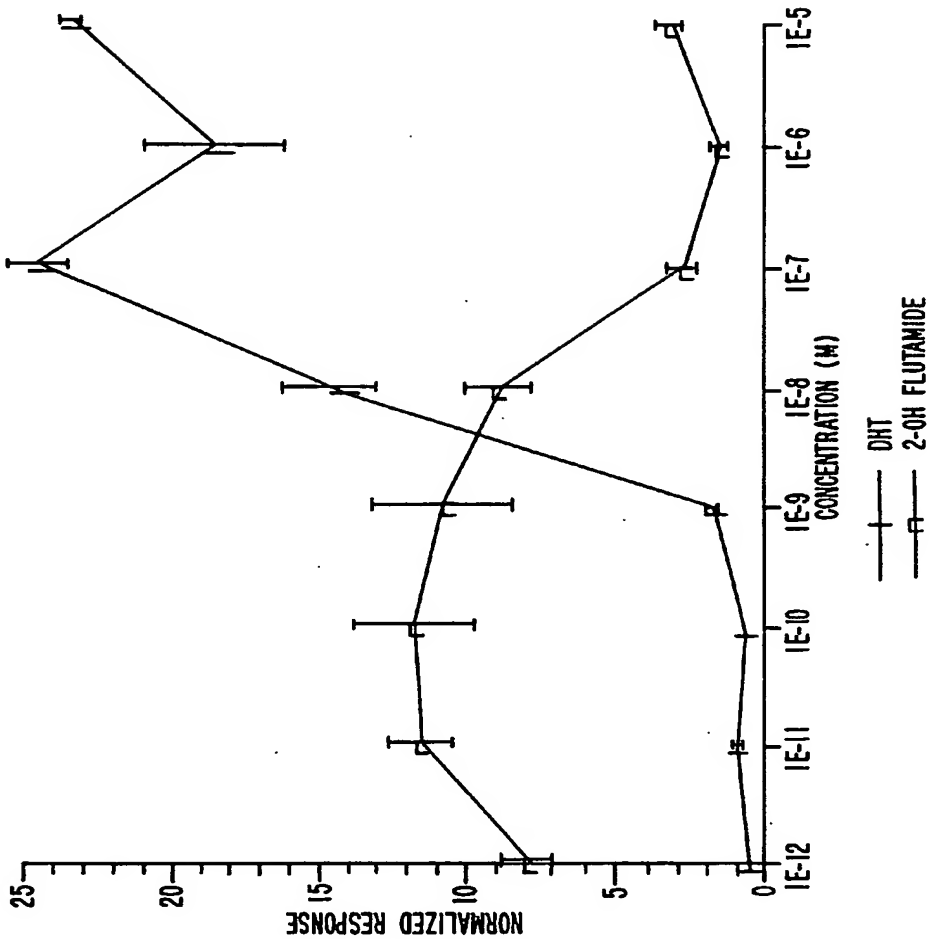
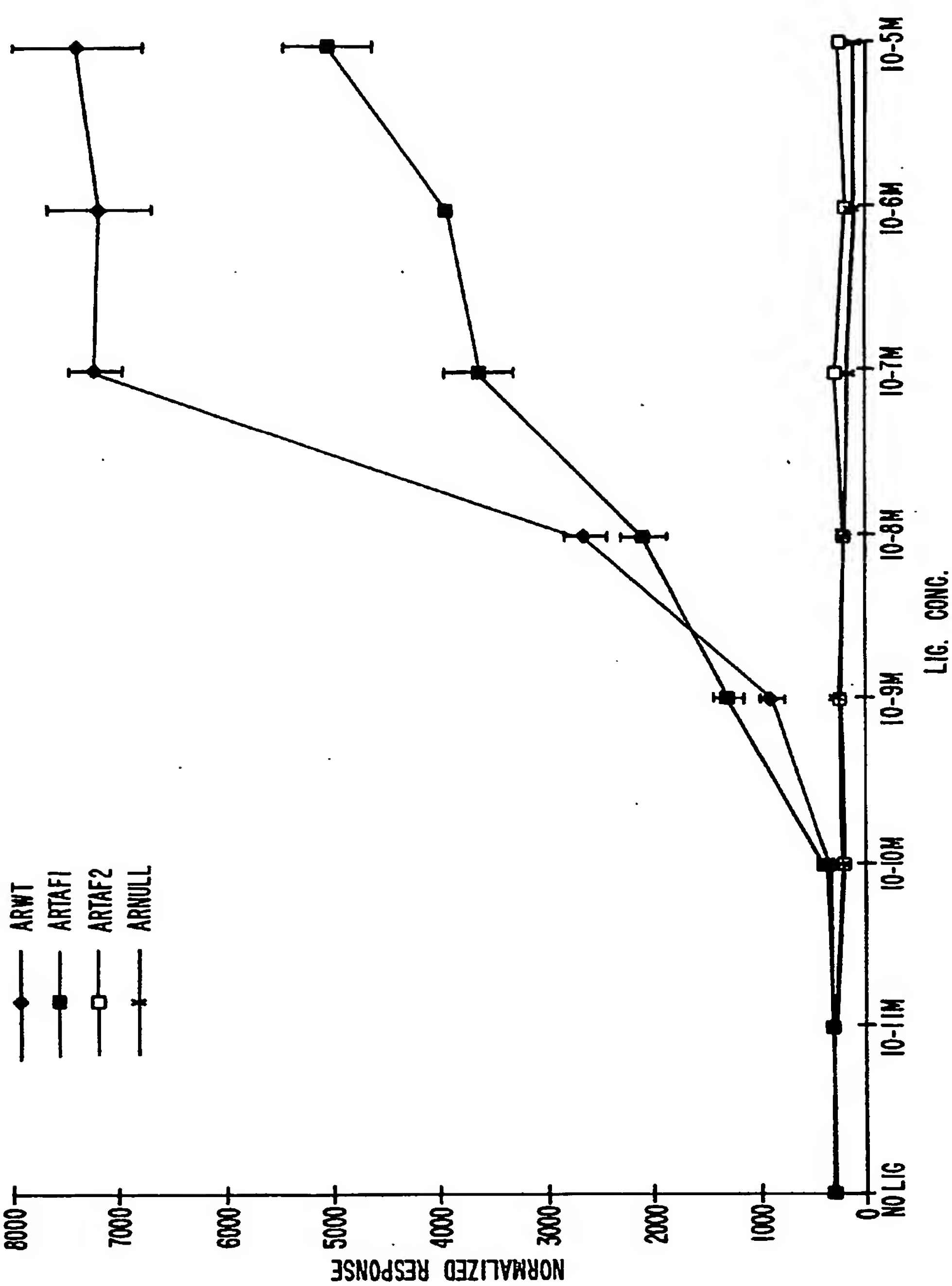

NORMALIZED RESPONSE
25
20
15
10
5
0
IE-12
IE-11
IE-10
IE-9
IE-8
IE-7
IE-6
IE-5
CONCENTRATION (M)
DHT
2-OH FLUTAMIDE

FIG. 18.


ARWT
ARTAF1
ARTAF2
ARNULL
NORMALIZED RESPONSE
8000
7000
6000
5000
4000
3000
2000
1000
0
NO LIG
10-11M
10-10M
10-9M
10-8M
10-7M
10-6M
10-5M
LIG. CONC.

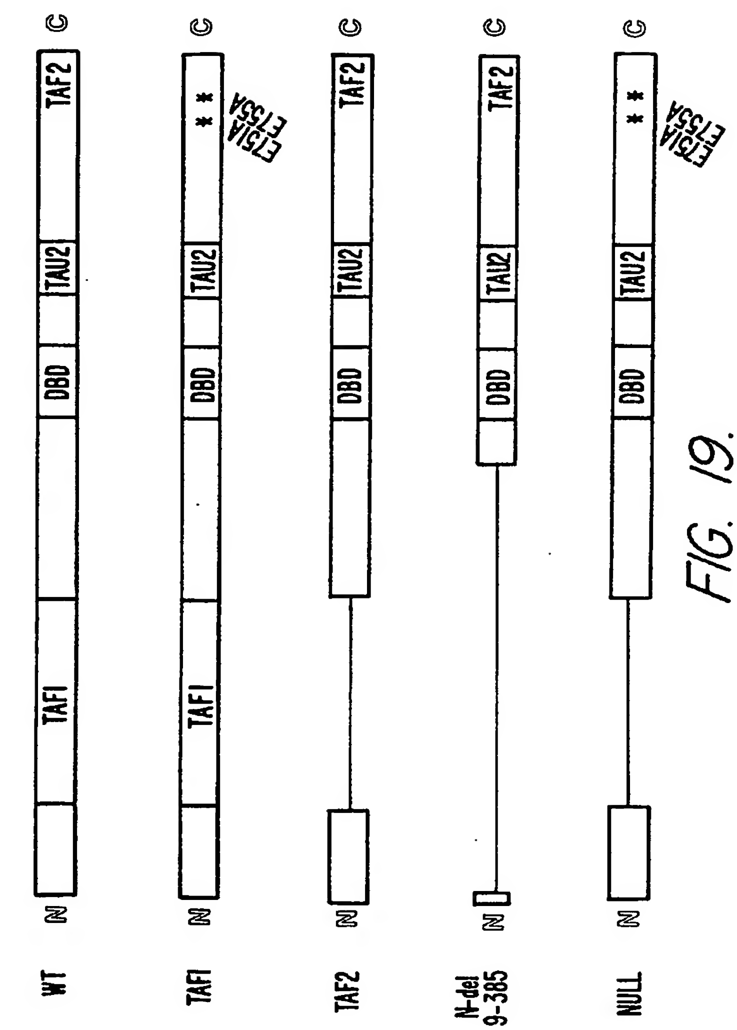


FIG. 19.

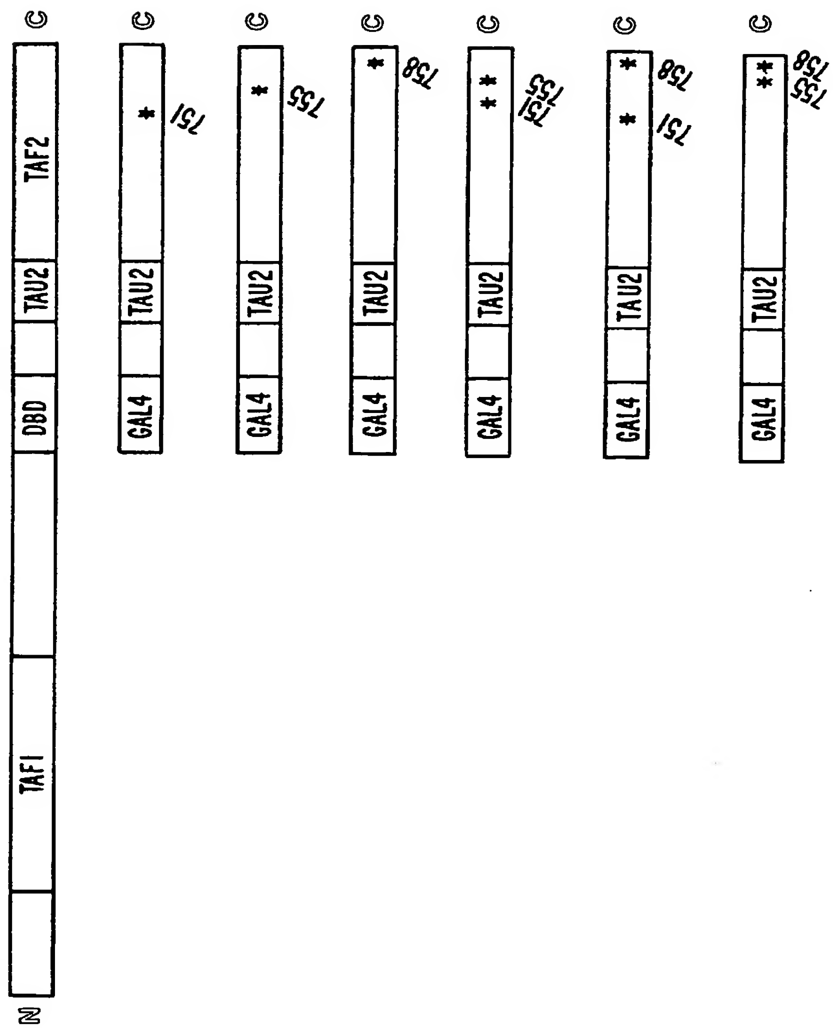


FIG. 20.

FIG. 21.

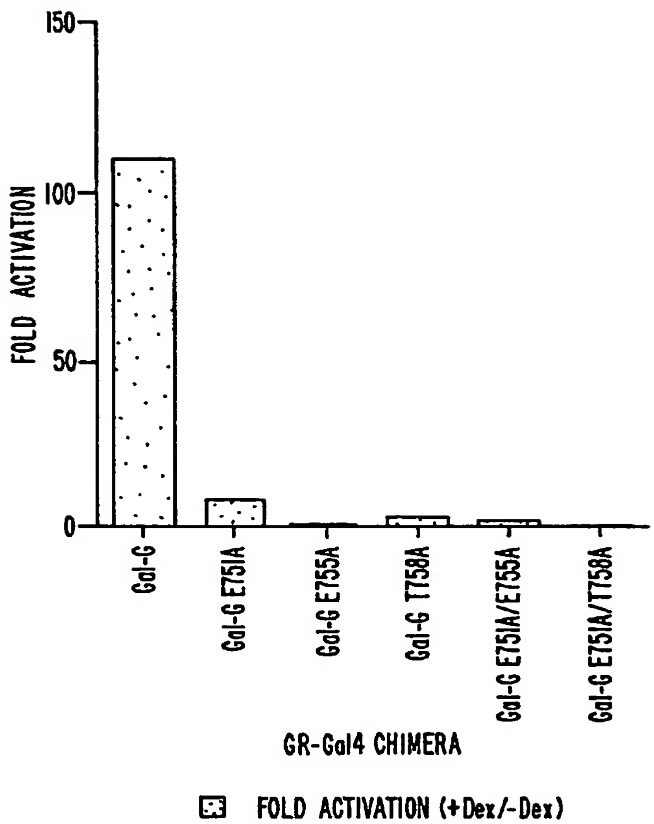
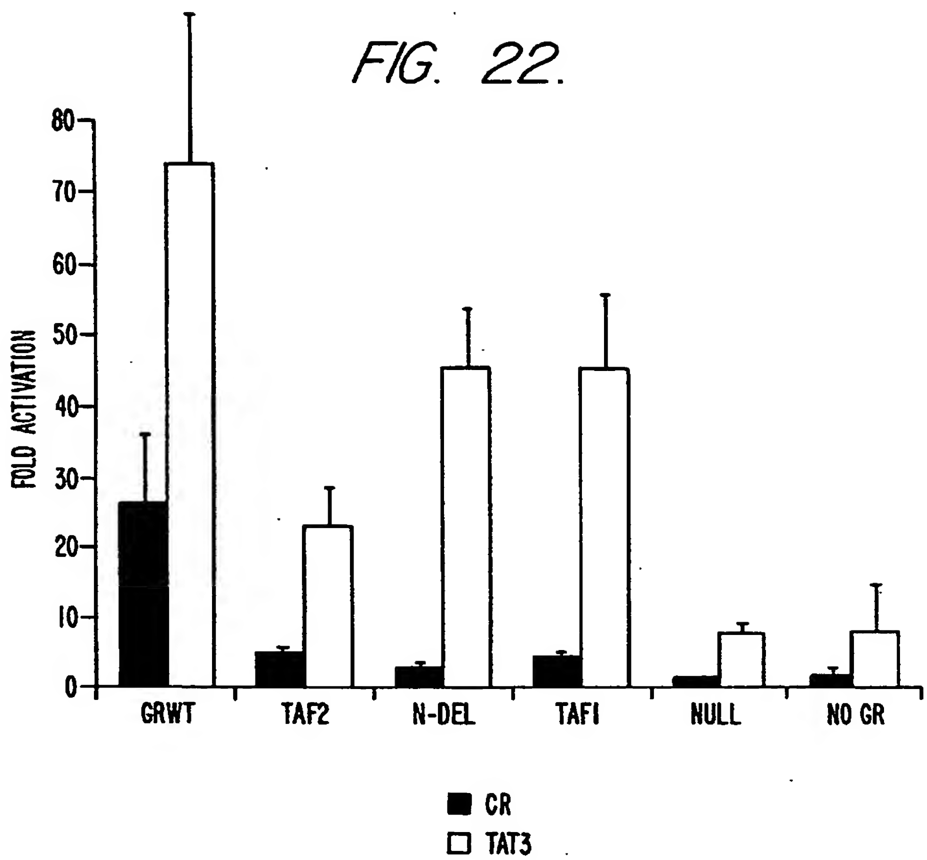

150
100
50
0
FOLD ACTIVATION
Gal-G
Gal-G E751A
Gal-G E755A
Gal-G T758A
Gal-G E751A/E755A
Gal-G E751A/T758A
GR-Gal4 CHIMERA
FOLD ACTIVATION (+Dex/-Dex)

FIG. 22.


FOLD ACTIVATION
80
70
60
50
40
30
20
10
0
GRWT
TAF2
N-DEL
TAFI
NULL
NO GR
CR
TAT3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09638

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N33/566 C12N15/12 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 23068 (LIGAND PHARM INC) 13 October 1994 see the whole document ---	1-21
Y	US,A,5 071 773 (EVANS RONALD M ET AL) 10 December 1991 cited in the application see the whole document ---	1-14
Y	BIO/TECHNOLOGY, vol. 11, November 1993, pages 1256-1261, XP002018219 MCDONNELL ET AL.: "Nuclear Hormone Receptors as Targets for New Drug Discovery" see the whole document ---	1-14
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 1996	27.11.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09638

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO J., vol. 11, no. 3, 1992, pages 1025-1033, XP002018220 DANIELIAN ET AL.: "Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormon receptors" cited in the application see the whole document ---	1-14
A	NUCL.ACID RES., vol. 17, 1989, pages 2581-2595, XP002018221 BOCQUEL ET AL.: "The contribution of the N-and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell-specific" see the whole document -----	1-14

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/09638

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-14: Method for identifying a receptor agonist or antagonist using a reporter-gene linked assay.
2. Claims 15-21: Method for treating a patient with an estrogen related disease using keoxifene-like compounds.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/09638

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9423068	13-10-94	AU-A-	6556194	24-10-94
		BR-A-	9406616	06-02-96
		CA-A-	2160135	13-10-94
		EP-A-	0694078	31-01-96
US-A-5071773	10-12-91	AU-B-	616389	31-10-91
		AU-A-	8238887	25-05-88
		EP-A-	0287653	26-10-88
		EP-A-	0733705	25-09-96
		JP-T-	1500964	06-04-89
		WO-A-	8803168	05-05-88
		US-A-	5534418	09-07-96
		US-A-	5312732	17-05-94
		US-A-	5298429	29-03-94